NRDC

April 1, 2019

SENT VIA CERTIFIED MAIL AND EMAIL TO:
guilaran.yu-ting@epa.gov, harty.thomas@epa.gov, hathaway.margaret@epa.gov, and synderman.steven@epa.gov.

Yu-Ting Guilaran, Director, Pesticide Re-evaluation Division
Steven Snyderman, Chemical Review Manager for Imidacloprid and Dinotefuran
Margaret Hathaway, Chemical Review Manager for Acetamiprid
Thomas Harty, Chemical Review Manager for Clothianidin and Thiamethoxam
U.S. Environmental Protection Agency
Office of Pesticide Programs, Mail Code 7506C
1200 Pennsylvania Ave., NW
Washington, D.C. 20460

RE: Imidacloprid Registration Review, Case No. 7605, EPA-HQ-OPP-2008-0844; Clothianidin Registration Review, Case No. 7620, EPA-HQ-OPP-2011-0865; Thiamethoxam Registration Review, Case No. 7614, EPA-HQ-OPP-2011-0581; Dinotefuran Registration Review, Case No. 7441, EPA-HQ-OPP-2011-0920; Acetamiprid Registration Review, Case No. 7617, EPA-HQ-OPP-2012-0329

Dear Ms. Guilaran, Ms. Hathaway, Mr. Harty, and Mr. Snyderman

I write on behalf of the Natural Resources Defense Council (NRDC) to notify you of three newly published studies that raise serious concerns about the ubiquity of neonicotinoid insecticides ("neonics") and their byproducts in drinking water and the potential mammalian toxicity of these compounds. *See* Kathryn L. Klarich Wong et al., *Chlorinated Byproducts of Neonicotinoids and their Metabolites: An Unrecognized Human Exposure Potential?*, Environ. Sci. Technol. Lett. 6(2), 98-105 (2019) (Att. 1); Tamanna Sultana et al., *Neonicotinoid Pesticides in Drinking Water in Agricultural Regions of Southern Ontario, Canada*, Chemosphere 202, 506-513 (March 2018) (Att. 2); Elise Hughes Berheim et al., *Effects of Neonicotinoid Insecticides on Physiology and Reproductive Characteristics of Captive Female and Fawn White-tailed Deer*, Scientific Reports 9:4354 (March 2019) (Att. 3). EPA must consider these studies in its pending registration reviews of imidacloprid, clothianidin, thiamethoxam, dinotefuran, and acetamiprid under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), 7 U.S.C. § 136 et seq.

As explained below, each study is directly relevant to EPA's human health risk assessments and tolerance reviews for all five neonics. Both drinking water studies demonstrate that neonics are routinely detected in drinking water and frequently survive conventional water treatment processes. Moreover, Klarich Wong et al. show toxic neonic degradates in treated drinking water and signal upcoming research that is likely to find additional, potentially toxic neonic chlorinated byproducts in treated drinking water. The third study, by Berheim et al., shows that imidacloprid can have wide-ranging impacts on endocrine, immune, and reproductive systems in mammals. Because neonics are good candidates for a cumulative risk assessment based on their similar

NATURAL RESOURCES DEFENSE COUNCIL

40 W 20TH STREET | NEW YORK, NY | 10011 | T 212.727.2700 | F 212.727.1773 | NRDC.ORG

chemical structures and pesticidal action in insects,[1] each study is relevant to all neonics currently under registration review.

Both FIFRA and EPA's implementing regulations require EPA to consider the most current information to ensure that the reviewed pesticide or pesticide class does not cause unreasonable adverse effects on the environment (the "FIFRA standard") before issuing a registration review decision. 40 C.F.R. § 155.40(a)(1) (stating that registration reviews are intended to ensure that pesticides meet the FIFRA standard "based on *current* scientific and other knowledge.") (emphasis added); *cf.* 7 U.S.C. § 136a(g)(1)(A) (requiring "periodic review" of registration every fifteen years to ensure pesticides meet the FIFRA standard). The comment periods for all neonic human health risk assessments closed prior to publication of the studies by Klarich Wong et al. (Jan. 2019) and Berheim et al. (Mar. 2019). Moreover, Sultana et al. published their study after the close of the comment period for EPA's human health risk assessment for imidacloprid.[2] EPA must not issue final registration decisions without considering the risks raised in these studies. *See* 40 C.F.R. § 155.40(a)(1). *West Harlem Environmental Action v. EPA*, 380 F. Supp. 2d 289, 295 n. 2 (S.D.N.Y. 2005) (re-registration of pesticide without requiring previous mitigation measures was arbitrary and capricious, in part, because EPA "inexplicably [] chose to ignore" data outside the administrative record that was "obvious[ly] . . . responsive to [a] key question").

To ensure EPA's registration review decision reflects current scientific knowledge, we urge EPA to publish addenda to its human health assessments or issue revised human health assessments. EPA should solicit public comment under either approach. EPA has previously used an addendum to supplement a completed human health assessment with new data. *See, e.g.*, Fluazianam; Addendum to the Human Health Risk Assessment for new Uses and Registration Review: Incorporation of New Acute Toxicity Data and Updated Restricted Entry Interval (Feb. 14, 2013). Moreover, EPA explained in the public notice of its Imidacloprid Human Health Assessment that the agency may publish a revised risk assessment before completing its review decision. Registration Review; Draft Human Health and/or Ecological Risk Assessments for Several Pesticides; Notice of Availability, 82 Fed. Reg. 43,006 (Sept. 13, 2017). EPA must undertake one of these established processes to ensure that EPA's registration review decision reflects current data on human health risks of neonic use.

---

[1] Thiamethoxam, clothianidin, imidacloprid, and dinotefuran all share a nitroguanidine grouping and all five neonics act as agonists of the nicotinic acetylcholine receptor in insects. D.B. Kanne et al., *Neonicotinoid Nitroguanidine Insecticide Metabolites: Synthesis and Nicotinic Receptor Potency of Guanidines, Aminoguanidines, and Their Derivatives*, Chem. Res. Toxicol. 18(19), 1479-84 (Sept. 2005). Abdessalam Kacimi El Hassani et al., *Effects of Sublethal Doses of Acetamiprid and Thiamethoxam on the Behavior of the Honeybee*, Arch Environ. Contam. Toxicol. 54:653-61 (2008); *see also* EPA, Guidance for Identifying Pesticide Chemicals and Other Substances that Have a Common Mechanism of Toxicity (Jan. 29, 1999) (hereafter "1999 CMT Guidance").

[2] EPA issued human health assessments for public comment on the following dates: Imidacloprid from September 13, 2017 to November 13, 2017; clothianidin, thiamethoxam, and dinotefuran from December 21, 2017 to April 21, 2018; and acetamiprid from Feb. 27, 2018 to April 30, 2018. NRDC submitted detailed comments on EPA's human health assessment for imidacloprid. Comments from the Natural Resources Defense Council on the Imidacloprid: Human Health Draft Risk Assessment for Registration Review, Doc. ID EPA-HQ-OPP-2008-0844-1235 (Nov. 13, 2017) (Att. 4).

Legal Background

I. The FQPA Requires EPA to Consider Risks Posed by Pesticide Degradates and Metabolites in Drinking Water.

The Food Quality Protection Act (FQPA) amended the Federal Food, Drug, and Cosmetic Act (FDCA) to require EPA to consider additional factors when establishing tolerances for pesticides on or in food. *See* 21 U.S.C. § 346a(b)(2)(D). It also incorporates tolerance review into FIFRA's registration procedures. *See* 7 U.S.C. § 136(bb). EPA may only establish a food tolerance for a pesticide if it determines that "there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information." 21 U.S.C. § 346a(a)(2)(A)(ii) (hereafter "FDCA standard"). EPA must consider "the aggregate exposure level of consumers . . . to the pesticide . . . and to other related substances," *id.* § 346a(a)(2)(D)(vi). This includes exposure to the pesticide and other related substances from all "other non-occupational sources," *id.*, including drinking water. *See, e.g.*, EPA, *Imidacloprid. Human Health Assessment Scoping Document in Support of Registration Review* 3 (Dec. 3, 2008) (explaining that exposure to imidacloprid in drinking water would be incorporated into the dietary assessment).

Degradates and metabolites of pesticides are among the "other related substances" which EPA must include in its tolerance assessment. *See, e.g.*, Proposed Order Granting Objections to Tolerances and Denying Request for a Stay, 76 Fed. Reg. 3,422, 3,442 (Jan. 19, 2011) (rejecting arguments that the tolerance assessment could not include fluoride, a degradate of the pesticide sulfuryl fluoride, added to drinking water in its aggregate risk assessment of the parent pesticide). Further, EPA must consider exposure to "other related substances," such as metabolites and degradates, through drinking water. Imidacloprid; Order Denying Objections to Issuance of Tolerance, 69 Fed. Reg. 30,042, 30,073 (May 26, 2004); *see also id.*, EPA Office of Pesticide Programs, *Estimating the Drinking Water Component of a Dietary Exposure Assessment* 18 (Nov. 2, 1999) ("[U]sefulness of the [water] monitoring data in a risk assessment" depends partly on "inclusion of important metabolites and degradates."). EPA has implicitly acknowledged its obligation to consider risks posed by pesticide degradates and metabolites in drinking water. *See* EPA, *Imidacloprid: Human Health Draft Risk Assessment for Registration Review* 22 (Jun. 22, 2017) (including imidacloprid urea, guanidine, and olefin in the water residue profile).

If EPA does not have "reasonable certainty" that aggregate pesticide exposure allowed by a tolerance will avoid all harm to human health, it must revoke the tolerance. 21 U.S.C. § 346a(a)(2)(A)(ii); *see also League of United Latin Am. Cit. v. Wheeler*, 899 F.3d 814, 829 (9th Cir. 2018) (*rehearing en banc granted*, 914 F.3d 1189 (9th Cir. 2019)) (holding EPA's own conclusion that there was "significant uncertainty" regarding effects of chlorpyrifos "mandates revoking the tolerance" for that chemical.).

In sum, when conducting a tolerance assessment for any pesticide, EPA must consider risks posed by degradates and metabolites of the parent pesticide compound if exposure to those substances results from any non-occupational source, including drinking water. If EPA cannot

conclude with "reasonable certainty" that the tolerance level in effect for a pesticide will not harm human health, EPA must reduce or revoke that tolerance.

II. EPA's Tolerance Assessment During Registration Review Must Reflect Current Science.

FIFRA requires EPA to reassess tolerances for a pesticide during the registration review process. *See* 40 C.F.R. § 155.40(a), 7 U.S.C. §136(bb). Registration review is designed to ensure that a registered pesticide does not cause "unreasonable adverse effects on the environment." 40 C.F.R. § 155.40(a). That standard includes "a human dietary risk from residues that result from a use of a pesticide in or on any food inconsistent with" the FDCA standard. 7 U.S.C. § 136(bb).

EPA must consider the most recent studies and data available during registration review. *See* 7 U.S.C. § 136a(c)(5), (g)(1)(A), 40 C.F.R. § 155.40(a)(1). The FIFRA registration process reflects Congress's intent that no pesticide should be used if it generally causes unreasonable adverse effects on the environment. *See generally* 7 U.S.C. § 136a(c)(5). Congress designed the registration review process to ensure that EPA's determination under that standard reflects the most recent information regarding pesticide risks. *Cf. id.* § 136a(g)(1)(A) (requiring "periodic review" of registration every fifteen years to ensure the pesticide satisfies the FIFRA standard). EPA's regulations reinforce this requirement. 40 C.F.R. § 155.40(a)(1) ("Registration review is intended to ensure that each pesticide's registration is based on current scientific and other knowledge regarding the pesticide, including its effects on human health and the environment.").

Both FIFRA and EPA's regulations, therefore, require EPA to consider during registration review the most recent information about environmental and human health implications of pesticide exposure. If EPA's registration review decision permits continued registration of a pesticide without addressing this information, EPA violates both FIFRA and the Administrative Procedure Act (APA), 5 U.S.C. § 706. *Cf. West Harlem Environmental Action*, 380 F. Supp. 2d at 295 n. 2 (re-registration of pesticide without requiring previous mitigation measures was arbitrary and capricious, in part, because EPA "inexplicably [] chose to ignore" data outside the administrative record that was "obvious[ly] . . . responsive to [a] key question").

Study Overviews

I. Klarich Wong et al. (2019)

Klarich Wong et al. (2019) report two overarching results that EPA must consider in its registration reviews. First, clothianidin, imidacloprid, and thiamethoxam, as well as two toxic metabolites of imidacloprid, were detected in finished drinking water. Second, potentially highly toxic chlorinated disinfection byproducts of all five substances can be formed through routine chlorination during water treatment. Moreover, the authors outline upcoming studies that are likely to lend further support to the hypothesis that people may be exposed to these chlorinated byproducts through treated drinking water. These results suggest that the risks associated with neonic exposure through drinking water are potentially far greater than EPA's health assessments suggest.

A. Traditional Water Treatment Methods Fail to Remove All Neonics from Drinking Water.

To assess the effectiveness of two water treatment methods in removing neonics and their byproducts, researchers collected three categories of water samples at two water treatment facilities: raw (pre-treatment), treated (immediately post-treatment), and tap (from the faucet of a home served by the treatment facility). Att. 1 at 4. The first facility (UI) "use[d] direct surface water and conventional coagulation/flocculation/sand-filtration with mixed powdered activated carbon during high dissolved organic matter (DOM) conditions (to control DBP formation)." *Id.* The second (IC) used "an alluvial well-field with granulated activated carbon (GAC) filter-beds." *Id.*[3]

Clothianidin, imidacloprid, and thiamethoxam were detected above the lower level of detection in all raw samples for both facilities. *Id.* at 6. All three neonics were also detected in UI tap water samples, with clothianidin and thiamethoxam detected in both treated and tap water from the UI plant. *Id.* at 7. Thiamethoxam was also detected in both treated and tap water at the IC plant. *Id.* These results demonstrate limited effectiveness of conventional water treatment methods, especially the method used at UI, in removing these substances. These results are largely consistent with an earlier study finding neonics in finished drinking water. *See* Klarich et al., *Occurrence of Neonicotinoids in Finished Drinking Water and Fate During Water Treatment*, Environ. Sci. Technol. Lett. 4(5) 168-73 (2017).

In addition to the parent compounds above, the researchers detected, for the first time, two imidacloprid degradates in drinking water. Att. 1 at 6. Desnitro imidacloprid was detectable in every sample and was present above the lower level of detection in six (6) of the nine (9) samples analyzed. *Id.* In tap water, desnitro imidacloprid was detected in concentrations between .03-.06 ng/L from the UI plant and less than .03 ng/L from the IC plant. *Id.* Further, imidacloprid urea was present at levels above the lower level of detection in five of nine total samples analyzed and was detected in UI tap water samples between .22 and .29 ng/L. *Id.*

Though these degradates were detected at low concentrations compared to the parent compounds considered in EPA's health assessments, desnitro imidacloprid is substantially more toxic than imidacloprid in mammals. *Id.* at 3 (desnitro imidacloprid is 317 times more toxic than imidacloprid based on IC50); *id.* at 7 ("Desnitro-imidacloprid has a substantially lower IC_{50} value than imidacloprid for vertebrates, indicating greater binding response (8.2 vs 2600 nM [1.7 vs 550 µg/L], respectively).").

The 2019 study, therefore, bolsters existing research showing that traditional water treatment methods are not completely effective in removing neonics from drinking water. It also shows for

[3] These two water treatment methods employ techniques that are representative of modern municipal water treatment. *See* Center for Disease Control, *Water Treatment: Community Water Treatment* (Jan. 20, 2015), https://bit.ly/2roz4Q2 (Coagulation, flocculation, sedimentation, filtration, and disinfection are the most common steps in modern water treatment.), EPA, *Drinking Water Treatability Database: Granular Activated Carbon*, https://bit.ly/2Wad5s1 ("Activated carbon is commonly used . . . in drinking water treatment" and the two main types are granular activated and powdered activated carbon.).

the first time that highly toxic degradates of imidacloprid are also present in finished drinking water. EPA must incorporate and analyze this new information as part of its tolerance analysis for each neonic.

B. Traditional Water Treatment Methods May Produce Chlorinated Disinfection Byproducts of Neonics in Drinking Water.

In addition to the water sampling described above, the authors assessed whether water treatment conditions could cause neonics and their degradates to transform into potentially highly toxic chlorinated disinfection byproducts. To do so, the researchers simulated water treatment conditions in a lab using pH adjustment and addition of free chlorine to the neonics detected in drinking water. Att. 1 at 5.

The authors found that both desnitro imidacloprid and imidacloprid urea degraded readily at chlorine concentrations and time periods typical of drinking water disinfection and distribution, indicating likely formation of chlorinated byproducts in finished drinking water under real-world conditions. *Id.* at 8. Unlike earlier estimates of the half-lives of other neonics in similar conditions, the authors determined that the half-life of desnitro imidacloprid can be measured in minutes. *Id.* Moreover, the study found that thiamethoxam hydrolyzed readily at pH levels comparable to those achieved through lime softening during water treatment. *Id.* at 11. Alkaline hydrolysis of thiamethoxam produced two compounds, one of which (THX-H 237) the study authors predict will react with chlorine at time-scales relevant to water treatment and distribution. *Id.*

The study authors then predicted chemical structures for the chlorinated byproducts of desnitro imidacloprid, imidacloprid urea, and thiamethoxam, as well as imidacloprid and clothianidin, which had previously been shown to be reactive with chlorine. They note that although the mammalian toxicity of these disinfection byproducts is not fully understood, several of the predicted chlorination byproducts exhibit structural changes that impact how the molecules bind with receptors. This could change the bioactivity of the molecules, and several "appear to lose the nitro-group through chlorination or hydrolysis, and/or gain one or more chlorines—both characteristics that may increase mammalian toxicity." *Id.* at 16.

In sum, the 2019 study demonstrates a high risk that commonly used water treatment methods may transform neonics into more toxic chlorinated byproducts. Though more research is needed to conclusively determine whether these byproducts occur in finished drinking water and to fully understand their toxicity, this research is underway. The authors predict that these byproducts may be significantly more toxic to humans than their parent compounds. EPA must consider the potentially substantial human health risks in its registration review assessments of neonics.

II. Sultana et al. (2018)

Sultana et al. (2018) conducted a similar study to determine the extent of neonic contamination of raw and treated drinking water in Canada. The researchers analyzed removal of neonics by water treatment facilities by using Polar Organic Chemical Integrative Samplers (POCIS) and grab samples at six facilities in rural Ontario. Att. 2 at 506. All facilities used "conventional

treatment systems, including coagulation, flocculation, sedimentation, filtration and chlorine disinfection." *Id.* at 512. The authors tested for imidacloprid, clothianidin, thiamethoxam, acetamiprid, thiacloprid, dinotefuran, and 5-hydroxy imidacloprid in both raw (pre-treatment) and treated drinking water at each facility. *Id.* at 507.

In grab samples, clothianidin, thiamethoxam, and imidacloprid were detected in raw drinking water at all six facilities. Both thiamethoxam and imidacloprid were also detected in treated drinking water at four and three of the treatment facilities, respectively. *Id.* at 510 (Table 3). Where detected, average concentrations of thiamethoxam in treated water at the six facilities ranged from 20.1 to 91.7 ng/L, while concentrations of imidacloprid in treated samples ranged from 2.4 to 4.8 ng/L. The authors note that peak concentrations in drinking water were likely missed, since the samples were taken in early July, but the pesticides are typically applied a month earlier. *Id.* at 512.

POCIS samples were used to determine time weighted average (TWA) concentrations of the neonics over a 13-15-day period for each treatment facility. This sampling method detected thiamethoxam, imidacloprid, clothianidin, thiacloprid, and acetamiprid in both raw and treated drinking water at three of the six facilities. *Id.* The study authors note that TWA concentrations were largely consistent with grab sample concentrations. *Id.* at 511. Moreover, they conclude that the results of the study are consistent with drinking water samples analyzed in Iowa by Klarich et al. in 2017. *See generally* Klarich (2017), *supra* p. 4.

III. Berheim et al. (2019)

In a third study, researchers analyzed the endocrine-disrupting effects of imidacloprid on white-tailed deer by administering aqueous imidacloprid through drinking water to captive does and fawns. Att. 3 at 1. The study authors compared water consumption, thyroid hormone function, behavioral responses, jawbone measurements, and imidacloprid concentrations in organs among four treatment groups: high, medium, and low imidacloprid exposure and an untreated control group. *Id.* at 1.

The study found that higher concentrations of imidacloprid in the spleen were correlated with higher mortality rates, *id.* at 7, and shorter jawbone lengths in fawns, *id.* at 6. Additionally, fawns with higher concentrations of imidacloprid in the spleen and genital organs were generally smaller and less healthy. *Id.* Concentrations of imidacloprid in fawn spleens was also inversely correlated with levels of free thyroxine hormones, likely causing lower metabolic rate and observed decreases in physical activity among affected fawns. *Id.* Water consumption was also inversely correlated with imidacloprid concentration, indicating that the deer avoided drinking water contaminated with imidacloprid. *Id.* at 8. However, imidacloprid was detected even in organs of the control group, showing the ubiquity of environmental contamination. *Id.* at 1.

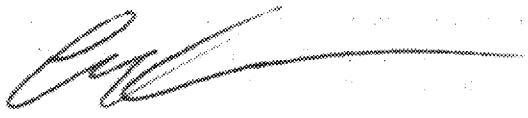
Berheim et al. demonstrate that imidacloprid exposure can result in a host of physiological effects in mammals, disrupting both endocrine function and immune response. *See id.* at 7. These findings are the latest in a growing body of reports linking neonic exposure with developmental and reproductive effects in non-target vertebrates, including birds and rodents, under field and laboratory conditions. *See, e.g.*, David Gibbons et al., *A Review of the Direct and Indirect Effects*

of Neonicotinoids and Fipronil on Vertebrate Wildlife, Environ Sci Pollut Res Int. 22(1), 103-18 (2015). This evidence undermines the central appeal of neonics—their supposed non-toxicity in humans and other mammals. Moreover, these observed toxic effects are relevant to EPA's determination of whether imidacloprid shares a common mechanism of toxicity with other neonics, as toxic effects in mammals are central to that analysis. *See* 1999 CMT Guidance, *supra* n. 1, at 7.

Conclusion

The three studies identified here bolster existing concerns about neonics in drinking water, show that neonic degradates are present in treated drinking water, raise new concerns that water treatment methods might produce chlorinated byproducts that are potentially more toxic to humans, and demonstrate toxic effects of neonics in mammals. FIFRA and the FQPA require EPA to consider this information in its evaluation of neonics' risks to human health. Moreover, FIFRA requires EPA to consider the most recent information available during its registration review process. EPA must not issue a final decision approving registration of neonics without addressing these substantial risks. We urge EPA to issue revised human health assessments or addenda to its previous assessments to ensure the attached studies are considered fully in the record for all neonics. Further, because all five neonics may share a common mechanism of toxicity, EPA should assess these human health risks for all five neonics cumulatively.

Respectfully submitted,



Lucas J. Rhoads
Staff Attorney, Nature Program
Natural Resources Defense Council
1152 15th St., N.W.
Washington, D.C. 20005
(646) 823-0472
lrhoads@nrdc.org

Environmental Science & Technology Letters

GEORGE MASON UNIVERSITY

Subscriber access provided by George Mason University Libraries & VIVA (Virtual Library of Virginia)

Environmental Processes

Chlorinated Byproducts of Neonicotinoids and their Metabolites: An Unrecognized Human Exposure Potential?

Kathryn L. Klarich Wong, Danielle T. Webb, Matthew R. Nagorzanski, Dana Ward Kolpin, Michelle L Hladik, David M. Cwiertny, and Gregory H. LeFevre

Environ. Sci. Technol. Lett., **Just Accepted Manuscript** • DOI: 10.1021/acs.estlett.8b00706 • Publication Date (Web): 14 Jan 2019

Environmental Science & Technology Letters

Chlorinated Byproducts of Neonicotinoids and their Metabolites: An Unrecognized Human Exposure Potential?

Kathryn L. Klarich Wong,[§,¶] *Danielle T. Webb,*[§,¶] Matthew R. Nagorzanski,[§,¶]

Dana W. Kolpin,[Δ] *Michelle L. Hladik,*[#] *David M. Cwiertny,*[§,¶,¥,€,*] *Gregory H. LeFevre*[§,¶,*]

[§]Department of Civil & Environmental Engineering, University of Iowa, 4105 Seamans Center, Iowa City, IA 52242, United States; [¶]IIHR-Hydroscience &Engineering, 100 C. Maxwell Stanley Hydraulics Laboratory, Iowa City, IA 52242, United States; [Δ]U.S. Geological Survey, Central Midwest Water Science Center, 400 S. Clinton St, Rm 269 Federal Building, Iowa City, IA 52240, United States; [#]U.S. Geological Survey, California Water Science Center, 6000 J Street, Placer Hall, Sacramento, CA 95819, United States; [¥]Center for Health Effects of Environmental Contamination, University of Iowa, 455 Van Allen Hall, Iowa City, Iowa 52242; [€]Public Policy Center, University of Iowa, 310 South Grand Ave, 209 South Quadrangle, Iowa City, IA 52242

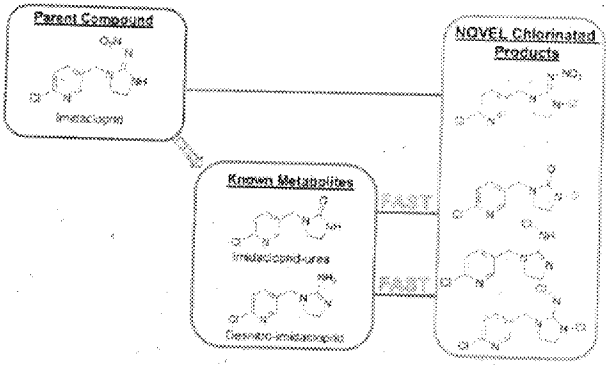
***Corresponding Authors:**
GHL: gregory-lefevre@uiowa.edu; Phone: 319-335-5655; 4105 Seamans Center for Engineering, University of Iowa, Iowa City IA, United States

DMC: david-cwiertny@uiowa.edu; Phone: 319-335-1401; 4105 Seamans Center for Engineering, University of Iowa, Iowa City IA, United States

ABSTRACT

We recently reported initial discovery of neonicotinoid pesticides in drinking water and potential for transformation through chlorination and alkaline hydrolysis during water treatment. The objectives of this research were to determine: (1) if neonicotinoid metabolites are relevant to drinking water exposure, and (2) the products formed from chlorination of neonicotinoids and their metabolites. Desnitro-imidacloprid and imidacloprid urea, two known metabolites of imidacloprid, are documented for the first time in drinking water. Desnitro-imidacloprid was present above the lower level of detection (0.03 ng/L) in 67% of samples (6/9) from drinking water systems but detectable in all samples (up to 0.6 ng/L). Although concentrations of desnitro-imidacloprid were lower than concentrations of parent neonicotinoids, desnitro-imidacloprid exhibits significantly more mammalian toxicity than imidacloprid. Using LC-HR-ToF-MS/MS analysis of laboratory experiments, we propose structures for novel transformation products resulting from the chlorination of clothianidin, imidacloprid, desnitro-imidacloprid, imidacloprid-urea, and hydrolysis products of thiamethoxam. Formation of chlorinated neonicotinoid byproducts occurs at timescales relevant to water treatment/distribution for the imidacloprid metabolites ($t_{1/2}$=2.4min-1.0h) and thiamethoxam hydrolysis products (4.8h). Imidacloprid metabolites in finished drinking water and potential formation of novel disinfection byproducts during treatment/distribution are relevant to evaluating the exposure and potential impacts of neonicotinoids on human health.

19 **TOC Art:**



20

INTRODUCTION

Neonicotinoids are the most widely used insecticides in the world.[1] Neonicotinoids are systemic, insect-targeting neurotoxins that have gained popularity due to their broad spectrum of control, high potency, and insect selectivity.[2–4] This insecticide class enjoys a wide range of both urban and agricultural uses, with a majority (~80% annually) of treated seeds planted in the United States coated with neonicotinoids.[1,5] Due to chemical properties (polarity, solubility) and heavy usage, neonicotinoids are commonly measured in surface waters across North America[6–10] with reported concentrations[7,11–14] up to 6900 ng/L. Neonicotinoid metabolites, such as desnitro-imidacloprid and imidacloprid-urea, are formed via microbial degradation, as well as some abiotic processes (*e.g.*, photolysis, hydrolysis).[2,3,5,15–22] As a result, these metabolites may also be present in surface waters used for drinking water.

Neonicotinoids exploit specific differences between nicotinic acetylcholine receptors (nAChR) in vertebrates and invertebrates to impart insect selectivity.[2,23] Neonicotinoids share important functional groups (nitroimines, cyanoimines, or nitromethylenes) to influence electrostatic binding potential; the negative polarity[24,25] on the neonicotinoid is rejected by the mammalian nAChR and readily accepted by the insect nAChR.[2] Although selective toxicity improves safety for non-target vertebrate organisms, the effects of chronic exposure of humans to neonicotinoids remain unknown.[26,27] Furthermore, toxicological profiles of neonicotinoid transformation products formed via degradation processes may be different from that of the parent compounds, particularly when the nitro- or cyano-groups are removed. For example, two known metabolites of imidacloprid and thiacloprid—desnitro-imidacloprid and descyano-thiacloprid—are respectively 317 and 195 times more toxic to mammals (based on IC_{50}) than their corresponding parent compounds.[3] Understanding the identity, fate, and bioactivity of transformation products

generated in natural and engineered systems is critical to understanding the full impacts of neonicotinoids on ecosystems and human health.

We recently reported[28] the first measurement of neonicotinoids in finished drinking water and demonstrated that select neonicotinoids can be transformed at elevated pH (thiamethoxam) or during chlorination (clothianidin, imidacloprid) over timescales relevant to water treatment and distribution. There is increasing concern about anthropogenic compounds acting as disinfection byproduct (DBP) precursors during disinfection[29] and the potential for these next-generation DBPs to exhibit retained or even enhanced bioactivity[30] (i.e., carcinogenic and/or genotoxic[31]). Objectives of this research were to determine: (1) if neonicotinoid metabolites are relevant to drinking water exposure, and (2) the products formed from chlorination of neonicotinoids and their metabolites that may be generated during drinking water treatment.

MATERIALS and METHODS

Drinking water samples. Raw and treated (entering and exiting treatment plant, respectively) drinking water samples were collected from the University of Iowa (UI) and Iowa City (IC) drinking water treatment plants (Iowa City, IA, USA). The treatment trains are detailed in the SI (Scheme S.1). The main similarities are both systems use lime softening at elevated pH (>10.3) and free chlorine disinfection, the main differences are that UI uses direct surface water and conventional coagulation/flocculation/sand-filtration with mixed powdered activated carbon during high dissolved organic matter (DOM) conditions (to control DBP formation), whereas IC uses an alluvial well-field with granulated activated carbon (GAC) filter-beds. Tap samples were collected from two buildings on the UI campus and three residences serviced by the IC plant

located throughout the city. The limited number of samples was intended to establish the presence and relevance of neonicotinoid metabolites in drinking water, but was not intended to be fully spatially / temporally representative, nor collected in a Lagrangian manner (i.e., transport time-adjusted). Samples were collected during the summer months, when neonicotinoid concentrations are highest.[6,28] UI and IC drinking water samples were analyzed for clothianidin, imidacloprid, thiamethoxam, desnitro-imidacloprid, and imidacloprid-urea. Methods for sample collection and analysis, as well as background information for both treatment and distribution systems, are described previously.[28] Analytical details, lower limits of detection (LLD), and field blank data are provided in the SI.

Hydrolysis, chlorination, and transformation product analysis. Fate during unit processes (lime softening, disinfection, and sequential lime softening and disinfection) was simulated in laboratory batch systems (described fully in the SI) using pH adjustment and free chlorine addition with neonicotinoid concentrations measured by liquid chromatography with diode array detector (LC-DAD). Experiments used free chlorine (HOCl) in a closed reactor containing 5 mM phosphate buffer (pH 7); a range of neonicotinoid (1–50 μM) and HOCl (1–50 mg/L) concentrations were tested (described in Figures S.2, S.3). Chlorination of thiamethoxam hydrolysis products occurred following initial hydrolysis at elevated pH with no chlorine (details in SI). Samples were monitored for 24-72 hours via LC-DAD, and then brought to the High-Resolution Mass Spectrometry Facility (HRMSF) at the University of Iowa for exact mass identification and MS/MS fragment analysis via LC-HR-ToF-MS/MS (Figures S6-S40). The Schymanski framework[32] was used for communicating confidence in identifying newly discovered small molecules (Table 1). Stability of chlorinated products (DN-IMI 245 chosen as representative example) was examined by adding

freshly-prepared sulfite (50 μM in the reactor) and observing back-transformation via LC-MS. Experimental details and analytical methods are provided in the SI.

RESULTS AND DISCUSSION

Occurrence of neonicotinoids and their metabolites in drinking water samples. Desnitro-imidacloprid was present above the Lower Level of Detection (LLD)[33] in 67% (6/9) of samples (raw, treated, and tap water) collected from UI (4/4) and IC (2/5) drinking water systems (Figure 1) but was detectable above the instrument signal-to-noise in all samples analyzed, representing the first known documentation of neonicotinoid metabolites in drinking water. The concentration of desnitro-imidacloprid ranged from <0.03-0.60 ng/L for all water samples. The desnitro-imidacloprid tap water concentrations ranged from 0.03-0.06 ng/L at UI and <0.03 ng/L for IC. Imidacloprid-urea was also present above the LLD in 56% (5/9) of all samples analyzed (4/4 for UI; 1/5 for IC), with measured detections ranging from 0.08-0.66 ng/L. Imidacloprid-urea was not detected in IC tap samples and ranged from 0.22-0.29 ng/L at UI taps (2/2). Clothianidin, imidacloprid, and thiamethoxam were also present in raw, treated, and tap samples with concentrations ranging from 2.34–25.34 ng/L for clothianidin, 1.02–8.79 ng/L for imidacloprid, and 0.24-5.99 ng/L for thiamethoxam. Notably, tap water concentrations for both UI and IC were similar to those we previously reported[28] (Table S.5). In contrast to our previous study, we observed removal of clothianidin and imidacloprid between the source and treated UI samples. We attribute removal to a powder activated carbon system that was added to UI for control of disinfection byproduct precursors after our initial study. This updated system is likely also removing neonicotinoid parent compounds, which we previously reported were effectively removed via activated carbon.[28]

Although the concentrations of metabolites were substantially lower than their respective parent compounds, select neonicotinoid metabolites are known to exhibit higher mammalian toxicity, based on limited available data. Desnitro-imidacloprid has a substantially lower IC_{50} value than imidacloprid for vertebrates, indicating greater binding response (8.2 vs 2600 nM [1.7 vs 550 µg/L], respectively).[3] The greater potential toxicity and frequent presence in these water samples of neonicotinoid metabolites demonstrates the need to consider their fate and persistence in drinking water treatment systems (*e.g.*, during chlorination and other treatment processes) and their potential effects on human health. Indeed, neonicotinoids have been measured year-round[10] in streams of impacted watersheds, and our results demonstrate that consumers of drinking water derived from vulnerable sources may be exposed to neonicotinoids and their metabolites.[28]

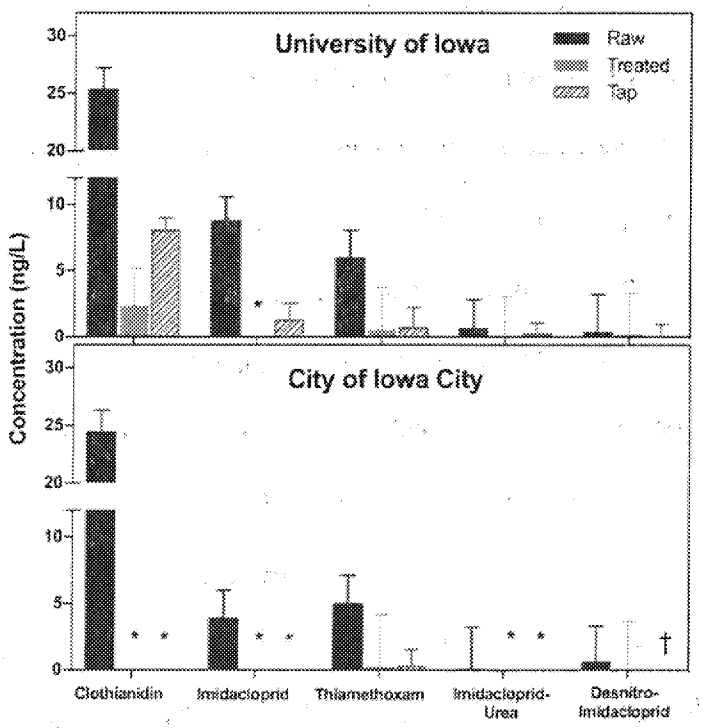


Figure 1: Clothianidin, imidacloprid, thiamethoxam, and two metabolites of imidacloprid (imidacloprid-urea and desnitro-imidacloprid) measured in raw and treated water from the University of Iowa and Iowa City water treatment plants (July 23 and 24, 2018, respectively). University of Iowa tap water was collected at two locations and Iowa City tap water was collected from three residences across Iowa City (n=2 and 3, respectively, July 17, 2018). Tap

126 concentrations are reported as averages (n=3, July 17, 2018), where (*) denotes non-detects while
127 (†) denote samples present below the lower detection limit (LLD). LLD values (ng/L):
128 clothianidin, 0.488; imidacloprid, 0.275; thiamethoxam, 0.081; desnitro-imidacloprid, 0.026;
129 imidacloprid-urea, 0.057. Error bars represent the standard error including the variation between
130 samples and in sample processing/analysis (associated with the composite enrichment, sample
131 extraction, and analysis).

132

133 **Desnitro-imidacloprid and Imidacloprid-urea Reactivity with Chlorine**. Desnitro-
134 imidacloprid and imidacloprid-urea react relatively rapidly during chlorination (Figure 2). Second-
135 order rate coefficients (±SE) for imidacloprid-urea (2.7±0.2 $M^{-1}s^{-1}$) and desnitro-imidacloprid
136 (72±5 $M^{-1}s^{-1}$) chlorination were calculated from measured pseudo-first-order rate constants (Figure
137 S1-S2) assuming a constant HOCl concentration during reaction ($k_2=k_{obs}/[HOCl]$). At a typical
138 chlorine concentration for disinfection (*i.e.*, 5 mg/L as Cl_2) and assuming a constant residual, half-
139 lives for imidacloprid-urea and desnitro-imidacloprid would be ~1.0 h and ~2.4 min, respectively.
140 As such, the metabolites of imidacloprid could be expected to degrade readily in a chlorine
141 contactor and during distribution.

142 Notably, the half-life of desnitro-imidacloprid is much shorter than those we previously
143 reported for clothianidin, imidacloprid, or thiamethoxam[28]—on the order of minutes compared to
144 hours or days for other neonicotinoids. We hypothesize that tautomerization[34] within the guanidine
145 functionality of desnitro-imidacloprid (Figure 2, Scheme S2) contributes to its greater reactivity,
146 resulting in an amino tautomer that would be expected to rapidly chloraminate based on the high
147 reactivity of primary amines toward free chlorine.[35] It remains unclear why imidacloprid-urea is
148 faster reacting than clothianidin and imidacloprid. Secondary and tertiary amides, such as those in
149 imidacloprid-urea, are known to be several orders of magnitude less reactive toward hypochlorous
150 acid than imine and guanidine analogs.[36] We therefore attribute the lower reactivity of clothianidin

and imidacloprid relative to imidacloprid-urea to the well-established electron-withdrawing nature of the nitro group.[37]

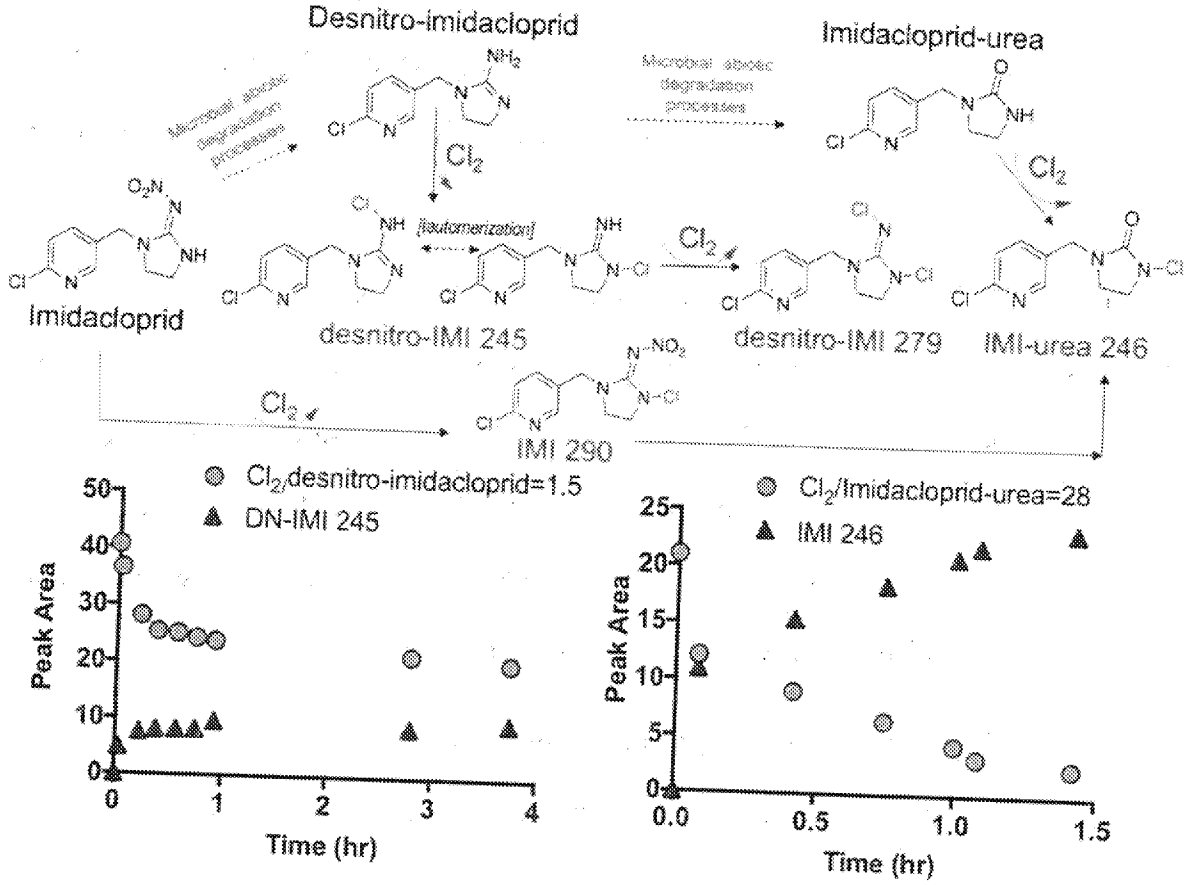
Using HR-MS/MS fragment analysis, we propose structures for byproducts observed during chlorination of desnitro-imidacloprid and imidacloprid urea. Chlorination of desnitro-imidacloprid results in the formation of two major identifiable products (hereafter desnitro-IMI 245 and desnitro-IMI 279), corresponding to the addition of either one or two chlorines (*i.e.*, the formation of one dichloro- and one trichloro-transformation product, respectively). Analysis of HR-MS/MS fragmentation patterns indicates chlorine addition occurring in the guanidine-containing portion of the molecule rather than the chloro-pyridine moiety (Fig S19) most likely via N-Cl bond formation; however, the exact site cannot be determined and thus desnitro-IMI 245 is reported at a Level 3 confidence.[32] Consistent with the formation of reactive N-Cl compounds, addition of excess sulfite to product mixtures after desnitro-imidacloprid chlorination resulted in the loss of detectable products and a corresponding increase in desnitro-imidacloprid (Figure S3). Such byproduct reversibility in the presence of a reducing agent is indicative of chloramine formation, as has been previously reported during chlorination of amine-containing pharmaceuticals.[38] Notably, this instability of desnitro-imidacloprid chlorination products may help to explain our detection of desnitro-imidacloprid in finished tap water (Figure 1) despite its very high reactivity toward free chlorine; decomposition of reactive byproducts could result in its regeneration during dechlorination with a reductant or via incidental reactions that occur within the distribution system.

We propose that desnitro-IMI 245 forms via chloramination of the amino tautomer of desnitro-imidacloprid (Figure 2, Scheme S2), which we expect to preferentially chlorinate prior to the corresponding imino tautomer based on established trends in the chlorination of structurally analogous N-containing compounds.[36,39] At higher chlorine concentrations or contact times, we

174 further hypothesize that sequential chlorination of desnitro-IMI-245 occurs through a chlorimino
175 derivative, where the added chlorine stabilizes the imino tautomer akin to the electron-withdrawing
176 nitro-group in imidacloprid. Although speculative, the secondary amine moiety in the chlorimino
177 tautomer would again be expected to exhibit greater reactivity toward chlorine than the
178 corresponding imine moiety in the chloramino tautomer.

179 Chlorination of imidacloprid-urea yielded one major identifiable product (hereafter IMI-urea
180 246). This corresponds to the addition of chlorine to the imidacloprid-urea structure. Once again,
181 HR-MS/MS fragment analysis is most consistent with chlorination occurring at the secondary
182 amide (Figure 2; Figures S39-S40).

183



184

Figure 2: Chlorination of (left) desnitro-imidacloprid and (right) imidacloprid-urea to form chlorinated products desnitro-IMI 245, desnitro-IMI 279 and IMI-urea 246. Chlorination kinetics of desnitro-imidacloprid to desnitro-IMI 245 and imidacloprid urea to IMI-urea 246 are shown. Peak area shown is the HPLC-DAD response λ= 260 nm for imidacloprid-urea; 273 nm for desnitro-IMI; relative values are shown because no authentic standards of chlorinated products are available. Initial concentration conditions (molar ratios shown in figures): desnitro-imidacloprid= 10μM, 1 mg/L HOCl as Cl_2; imidacloprid urea= 5μM, 1 mg/L HOCl as Cl_2. Full kinetics data, conditions in Figures S1, S2.

Hydrolysis Products of Thiamethoxam and Reactivity with Chlorine. The alkaline hydrolysis of thiamethoxam (at pH 10; relevant to lime softening) results in two products (hereafter THX-H 248 and THX-H 237), both of which have been previously identified with proposed pathways.[5,15,20,40] Imines are known to easily hydrolyze in water to yield ketones,[41,42] and the electron-withdrawing -NO_2 substituent makes the carbon in the guanidine portion of thiamethoxam more electrophilic, thus inviting hydroxide attack under alkaline conditions.[20] THX-H 248 is formed through the simple hydrolysis of the nitro-imine group into a ketone.[20] THX-H 237 was reported by Maienfisch[5] and corresponds to a ring opening with hydroxide attack at the imine carbon.

Upon addition of chlorine, THX-H 237 is reactive, while THX-H 248 is recalcitrant over the timescales / conditions investigated (Figure 3). We attribute the greater reactivity of THX-H 237 toward chlorine to the presence of its two secondary amides. The second-order rate coefficient (±SE) for the reaction of free chlorine with THX-H 237 (0.67±0.02 $M^{-1}s^{-1}$) was calculated from the measured pseudo-first-order rate constant (Figure S4). Assuming a constant chlorine residual (5 mg/L Cl_2), the half-life of THX-H 237 would be 4.8 h.

THX-H 237 reacts with chlorine to produce a single species hereafter referred to as CLO-THX-H 270 (see also Table 1). We propose that chlorine addition occurs at the secondary amide group

without the electron-withdrawing nitro substituent (Figure 3). Our MS/MS fragmentation results reveal a corresponding chlorinated fragment to support this proposed structure (Figure S9-S10). We anticipate that THX-H 237 will react to generate CLO-THX-H 270 at time-scales relevant to disinfection and distribution in systems that also employ chemical (e.g., lime-soda) softening earlier in the treatment process train.

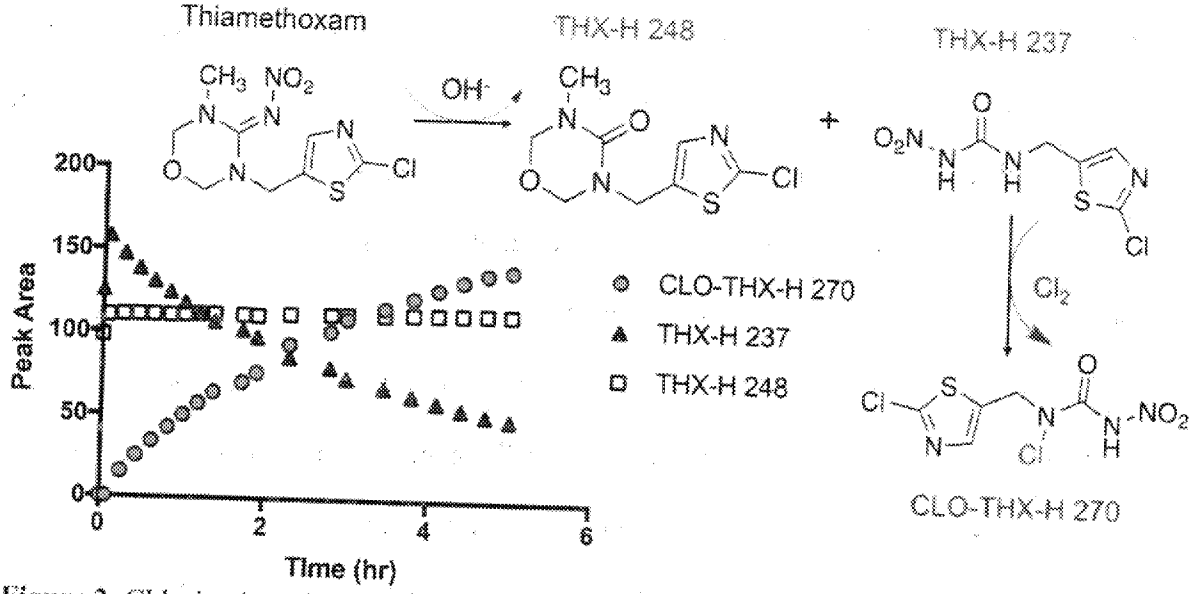


Figure 3: Chlorination of the hydrolysis products of thiamethoxam (THX-H 237 and THX-H 248) to form novel chlorinated product CLO-THX-H 270. Chlorination kinetics (represented by HPLC-DAD peak area λ = 260 nm; authentic standards unavailable; 50 mg/L Cl_2) of thiamethoxam hydrolysis product THX-H 237 to CLO-THX 270 is shown (THX-H 248 was unreactive) at pH 10. The structure of CLO-THX 270 (the same as generated through chlorination of clothianidin) is presented as shown to be consistent with Table 1; chlorination occurs at either amine farther from the nitro-group as determined by HR-MS/MS fragmentation (Figures S9, S10, S34, S35).

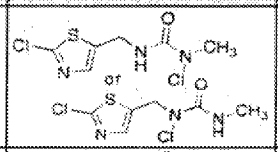
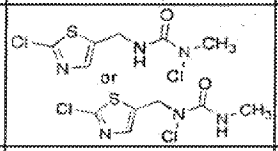
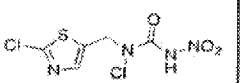
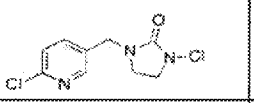
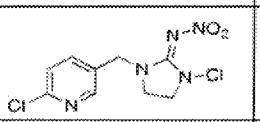
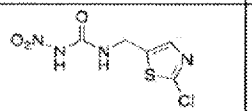
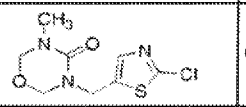
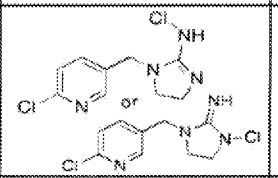
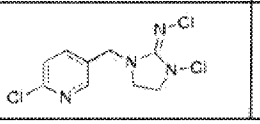
Products of Imidacloprid and Clothianidin Chlorination. We previously reported timescales for the reaction of imidacloprid and clothianidin with chlorine.[28] Herein, we propose structures using the Schymanski framework[32] to communicate confidence of novel products discovery for

229 the products of these reactions (Table 1) based on HR-MS/MS fragment analysis of these product
230 mixtures (Table S.7 describes compounds prior to chlorination).

231 Chlorination of clothianidin results in three major products. Two products have with the same
232 mass (hereafter CLO-239a and CLO-239b) but different retention times, while the third has an
233 exact mass $[M+H]^+$ of 270.9442. The latter product appears identical to the product formed during
234 chlorination of thiamethoxam hydrolysis products, and is thus also referred to as CLO-THX-H
235 270. Clothianidin is a known product of thiamethoxam degradation through multiple reported
236 biologically-mediated pathways[43,44] (e.g., in insects, mammals, plants, and soil) where the two
237 compounds share common metabolites;[25,45] however, abiotic and biological pathways may
238 generate different products. CLO 239a and CLO 239b correlate to loss of the nitro group,
239 formation of the ketone (C=O), and chlorination of a remaining secondary amide. We suspect these
240 reactions occur in a step-wise fashion and involve both oxidation with chlorine and hydrolysis
241 (*e.g.*, imine hydrolysis to a ketone) reactions, potentially involving intermediates we were unable
242 to identify. The exact location of the chlorine on two of the clothianidin products (CLO 239a, CLO
243 239b) could not be confirmed with certainty because MS/MS fragmentation did not yield the
244 chlorinated component (Figure S11-S14; Level 3 confidence). Nevertheless, chlorination is most
245 likely to occur at either of the secondary amides because HR-MS/MS fragment analysis indicated
246 that the chlorothiazole component was not further chlorinated (Figure S8-S14). Fragmentation
247 analysis of CLO-THX-H 270 generated either with clothianidin or thiamethoxan as the parent
248 compounds suggests that chlorination occurs at the nitrogen farther from the nitro group because
249 a chlorinated fragment consistent with this structure was present (Figures S9, S10; S34, S35; Level
250 2b confidence).

Chlorination of imidacloprid forms three major transformation products (hereafter: IMI-urea 246, IMI 290, and IMI 341). Product IMI-urea-246 is chlorinated imidacloprid-urea, which we previously identified in our independent analysis of products generated from the chlorination of an imidacloprid-urea standard (described above). IMI 290 is chlorinated imidacloprid (without loss of the nitro group), with chlorination most likely occurring at the secondary nitrogen in its guanidine moiety. One product, IMI 341, could only be confirmed to level 5 confidence,[32] thus no structure is proposed.

Table 1: Transformation products of clothianidin, imidacloprid, desnitro-imidacloprid, imidacloprid-urea, and thiamethoxam.

	Neonicotinoid Chlorination and Hydrolysis Transformation Products						Fragment Ions	
Parent Compound	Product Name	Proposed Structure	Proposed Formula	Schymanski Confidence Level‡	RT (min)	Accurate Mass $[M+H]^+$	Accurate mass (m/z)	Proposed Molecular Formula
Clothianidin	CLO 239 a		$C_6H_7Cl_2N_5OS$	Level 3	16.1	239.9792	168.0261	C_3H6N_3OS
							174.9774	$C_5H_4ClN_2OS$
							204.0124	$C_6H_7ClN_3OS$
							119.9693	C_2H_2ClNS
							86.0095	C_3H_3NS
Clothianidin	CLO 239 b		$C_6H_7Cl_2N_5OS$	Level 3	16.4	239.9798	174.9771	$C_5H_4ClN_2OS$
							146.982	$C_4H_4ClN_2S$
							131.9711	C_4H_4ClNS
							168.0261	$C_6H_6N_3OS$
							119.9788	C_2H_3ClNS
Clothianidin, THX-H 237	CLO-THX-H 270		$C_3H_4Cl_2N_4O_3S$	Level 2b	9.2	270.9442	181.9439	$C_4H_3Cl_2N_2S$
							146.9768	$C_4H_3ClN_2S$
							132.9717	C_4H_3ClNS
							118.9552	C_3HClNS
Imidacloprid, Imidacloprid-urea	IMI-urea 246		$C_9H_9Cl_2N_3O$	Level 2b	15.9	246.0222	211.0487	$C_9H_{10}ClN_3O$
							155.0348	$C_7H_7ClN_2$
							141.0206	$C_6H_6ClN_2$
							126.0097	C_6H_5ClN
Imidacloprid	IMI 341	Unknown	Ambiguous	Level 5	16.6	341.9938	218.0239	Unknown
							155.0367	Unknown
							126.0104	Unknown
Imidacloprid	IMI 290		$C_9H_9Cl_2N_5O_2$	Level 2b	16.9	290.0222	246.0217	$C_9H_9Cl_2N_3O$
							209.0617	$C_9H_{10}ClN_3$
							173.0839	$C_9H_{10}N_3$
							126.0123	C_6H_5ClN
Thiamethoxam	THX-H 237		$C_5H_5ClN_4O_3S$	Level 2b	11.8	236.9838	174.9724	$C_5H_5ClN_2OS$
							147.9772	$C_4H_5ClN_2S$
							97.0388	C_4H3NS
Thiamethoxam	THX-H 248		$C_8H_{10}ClN_5O_3S$	Level 2a	11.2	248.0248	174.9718	$C_5H_4ClN_2OS$
							98.0048	C_4H_3NS
							131.9665	C_4H_3ClNS
Desnitro-imidacloprid	desnitro-IMI 245		$C_9H_{10}Cl_2N_4$	Level 3	14.7	245.0377	209.0622	$C_9H_{10}ClN_4$
							173.0848	$C_9H_{10}N_3$
							211.0766	$C_9H_{12}ClN_4$
							83.0588	$C_3H_5N_3$
							132.0353	$C_4H_3ClN_3$
							126.0133	C_6H_5ClN
Desnitro-imidacloprid	desnitro-IMI 279		$C_9H_9Cl_3N_4$	Level 2b	18.5	279.0004	209.0506	$C_9H_{10}ClN_4$
							173.0848	$C_9H_{10}N_3$
							126.0130	C_6H_5ClN

‡The confidence level and structure of each product is characterized according to the Schymanski et al. 2014 framework for identifying small molecules via high resolution mass spectrometry.[32] All samples were analyzed in in ESI positive mode (i.e., ion $[M+H]^+$ =compound exact mass+H). High-resolution fragmentation patterns are presented in Figures S6-S40.

Environmental Implications. This is the first known study to report neonicotinoid metabolites in drinking water, and builds upon our prior research[28] and a subsequent publication from Canada[46] demonstrating neonicotinoids in drinking water. We also show that neonicotinoids and their known metabolites can form transformation products during disinfection and/or lime softening (hydrolysis at elevated pH) at timescales relevant to water treatment / distribution. The mammalian toxicity of transformation products formed during water treatment processes remains unknown. It is possible that chlorination of neonicotinoids and their metabolites will impact receptor binding interactions and alter their bioactivity relative to that of the parent neonicotinoids or known metabolites, a scenario that requires further investigation. Several transformation products identified (CLO 239a, CLO 239b, CLO-THX-H 270, IMI 246, THX-H 248, DN-IMI 245 and DN-IMI 279) appear to lose the nitro-group through chlorination or hydrolysis, and/or gain one or more chlorines—both characteristics that may increase mammalian toxicity.[3,4,23,29,31,47] Additional studies are needed to better assess temporal and spatial trends in metabolite occurrence / toxicity of chlorinated DBPs formed during drinking water treatment (including synthesized standards), especially in waters impacted by parent neonicotinoid insecticides.

SUPPORTING INFORMATION. Additional method details, statistical analysis, quality assurance / control, additional detailed data / results / analysis in figures and tables.

AUTHOR INFORMATION.

*Corresponding Authors:
GHL: gregory-lefevre@uiowa.edu; Phone: 319-335-5655; 4105 Seamans Center for Engineering, University of Iowa, Iowa City IA, United States

DMC: david-cwiertny@uiowa.edu; Phone: 319-335-1401; 4105 Seamans Center for Engineering, University of Iowa, Iowa City IA, United States

285 **NOTES.** The authors declare no competing financial interest.

286

287 **ACKNOWLEDGEMENT.**

288 This work was supported by grants from the University of Iowa Center for Health Effects of
289 Environmental Contamination (CHEEC), the National Science Foundation (CBET Environmental
290 Engineering 1803197), and through programmatic support of the U.S. Geological Survey Toxic
291 Substances Hydrology Program. KLK was supported by National Science Foundation Graduate
292 Research Fellowship Program (NSF GRFP). DTW was supported by the University of Iowa Center
293 for Biocatalysis and Bioprocessing / NIH Predoctoral Training Program in Biotechnology (2 T32
294 GM008365) and the Alfred P. Sloan Foundation Sloan Center for Exemplary Mentoring. We thank
295 Scott Slee at the University of Iowa Water Treatment plant and Kevin Slutts, Jon Durst, and Craig
296 Meacham at the Iowa City Water Treatment plant and Lynn Teesch and Vic Parcell at the UI
297 HRMS Facility. Any use of trade, firm, or product names is for descriptive purposes only and does
298 not imply endorsement by the U.S. Government.

299

300 **REFERENCES.**

301 (1) Jeschke, P.; Nauen, R.; Schindler, M.; Elbert, A. Overview of the Status and Global Strategy
302 for Neonicotinoids. *J. Agric. Food Chem.* **2011**, *59* (7), 2897–2908 DOI:
303 10.1021/jf101303g.

304 (2) Tomizawa, M.; Lee, D. L.; Casida, J. E. Neonicotinoid Insecticides: Molecular Features
305 Conferring Selectivity for Insect versus Mammalian Nicotinic Receptors. *J. Agric. Food*
306 *Chem.* **2000**, *48* (12), 6016–6024 DOI: 10.1021/jf000873c.

307 (3) Tomizawa, M.; Casida, J. E. Neonicotinoid Insecticide Toxicology: Mechanisms of
308 Selective Action. *Annu. Rev. Pharmacol. Toxicol.* **2005**, *45* (1), 247–268 DOI:
309 10.1146/annurev.pharmtox.45.120403.095930.

310 (4) Tomizawa, M.; Casida, J. E. Unique Neonicotinoid Binding Conformations Conferring
311 Selective Receptor Interactions. *J. Agric. Food Chem.* **2010**, *59* (7), 2825–2828.

(5) Maienfisch, P. Synthesis and Properties of Thiamethoxam and Related Compounds. *Zeitschrift für Naturforsch. B* **2006**, *61* (4), 353–359 DOI: 10.1515/znb-2006-0401.

(6) Hladik, M. L.; Kolpin, D. W.; Kuivila, K. M. Widespread Occurrence of Neonicotinoid Insecticides in Streams in a High Corn and Soybean Producing Region, USA. *Environ. Pollut.* **2014**, *193*, 189–196 DOI: 10.1016/j.envpol.2014.06.033.

(7) Hladik, M. L.; Kolpin, D. W. First National-Scale Reconnaissance of Neonicotinoid Insecticides in Streams across the USA. *Environ. Chem.* **2016**, *13* (1), 12 DOI: 10.1071/EN15061.

(8) Main, A. R.; Michel, N. L.; Headley, J. V.; Peru, K. M.; Morrissey, C. A. Ecological and Landscape Drivers of Neonicotinoid Insecticide Detections and Concentrations in Canada's Prairie Wetlands. *Environ. Sci. Technol.* **2015**, *49* (14), 8367–8376 DOI: 10.1021/acs.est.5b01287.

(9) Main, A. R.; Headley, J. V.; Peru, K. M.; Michel, N. L.; Cessna, A. J.; Morrissey, C. A. Widespread Use and Frequent Detection of Neonicotinoid Insecticides in Wetlands of Canada's Prairie Pothole Region. *PLoS One* **2014**, *9* (3), e92821 DOI: 10.1371/journal.pone.0092821.

(10) Hladik, M. L.; Corsi, S. R.; Kolpin, D. W.; Baldwin, A. K.; Blackwell, B. R.; Cavallin, J. E. Year-Round Presence of Neonicotinoid Insecticides in Tributaries to the Great Lakes, USA. *Environ. Pollut.* **2018**, *235*, 1022–1029 DOI: https://doi.org/10.1016/j.envpol.2018.01.013.

(11) Hladik, M. L.; Kolpin, D. W.; Kuivila, K. M. Widespread Occurrence of Neonicotinoid Insecticides in Streams in a High Corn and Soybean Producing Region, USA. *Environ. Pollut.* **2014**, *193*, 189–196 DOI: 10.1016/j.envpol.2014.06.033.

(12) Van Metre, P. C.; Alvarez, D. A.; Mahler, B. J.; Nowell, L.; Sandstrom, M.; Moran, P. Complex Mixtures of Pesticides in Midwest U.S. Streams Indicated by POCIS Time-Integrating Samplers. *Environ. Pollut.* **2017**, *220*, 431–440 DOI: 10.1016/j.envpol.2016.09.085.

(13) Evelsizer, V.; Skopec, M. Pesticides, Including Neonicotinoids, in Drained Wetlands of Iowa's Prairie Pothole Region. *Wetlands* **2016**, 1–12 DOI: 10.1007/s13157-016-0796-x.

(14) Starner, K.; Goh, K. S. Detections of the Neonicotinoid Insecticide Imidacloprid in Surface Waters of Three Agricultural Regions of California, USA, 2010–2011. *Bull. Environ. Contam. Toxicol.* **2012**, *88* (3), 316–321 DOI: 10.1007/s00128-011-0515-5.

(15) Todey, S. A.; Fallon, A. M.; Arnold, W. A. Neonicotinoid Insecticide Hydrolysis and Photolysis: Rates and Residual Toxicity. *Environ. Toxicol. Chem.* **2018**, *37* (11), 2797–2809 DOI: 10.1002/etc.4256.

(16) Zheng, W.; Liu, W. Kinetics and Mechanism of the Hydrolysis of Imidacloprid. *Pestic. Sci.* **1999**, *55* (4), 482–485 DOI: 10.1002/(SICI)1096-9063(199904)55:4<482::AID-PS932>3.0.CO;2-3.

(17) Bonmatin, J.-M.-M.; Giorio, C.; Girolami, V.; Goulson, D.; Kreutzweiser, D. P.; Krupke, C.; Liess, M.; Long, E.; Marzaro, M.; Mitchell, E. A. D.; Noome, D. A.; Simon-Delso, N.;

352 Tapparo, A. Environmental Fate and Exposure; Neonicotinoids and Fipronil. *Environ. Sci.*
353 *Pollut. Res.* **2015**, *22* (1), 35–67 DOI: 10.1007/s11356-014-3332-7.

354 (18) Lu, Z.; Challis, J. K.; Wong, C. S. Quantum Yields for Direct Photolysis of Neonicotinoid
355 Insecticides in Water: Implications for Exposure to Nontarget Aquatic Organisms. *Environ.*
356 *Sci. Technol. Lett.* **2015**, *2* (7), 188–192 DOI: 10.1021/acs.estlett.5b00136.

357 (19) Gusvany, V.; Csanadi, J.; Gaal, F. NMR Study of the Influence of PH on the Persistence of
358 Some Neonicotinoids in Water. *Acta Chim. Slov.* **2006**, *53*, 52–27.

359 (20) Karmakar, R.; Singh, S. B.; Kulshrestha, G. Kinetics and Mechanism of the Hydrolysis of
360 Thiamethoxam. *J. Environ. Sci. Heal. Part B* **2009**, *44* (5), 435–441 DOI:
361 10.1080/03601230902934785.

362 (21) Simon-Delso, N.; Amaral-Rogers, V.; Belzunces, L. P.; Bonmatin, J. M.; Chagnon, M.;
363 Downs, C.; Furlan, L.; Gibbons, D. W.; Giorio, C.; Girolami, V.; Goulson, D.;
364 Kreutzweiser, D. P.; Krupke, C. H.; Liess, M.; Long, E.; McField, M.; Mineau, P.; Mitchell,
365 E. A. D.; Morrissey, C. A.; Noome, D. A.; Pisa, L.; Settele, J.; Stark, J. D.; Tapparo, A.;
366 Van Dyck, H.; Van Praagh, J.; Van der Sluijs, J. P.; Whitehorn, P. R.; Wiemers, M. Systemic
367 Insecticides (Neonicotinoids and Fipronil): Trends, Uses, Mode of Action and Metabolites.
368 *Environ. Sci. Pollut. Res. Int.* **2015**, *22* (1), 5–34 DOI: 10.1007/s11356-014-3470-y.

369 (22) Aregahegn, K. Z.; Shemesh, D.; Gerber, R. B.; Finlayson-Pitts, B. J. Photochemistry of
370 Thin Solid Films of the Neonicotinoid Imidacloprid on Surfaces. *Environ. Sci. Technol.*
371 **2017**, *51* (5), 2660–2668 DOI: 10.1021/acs.est.6b04842.

372 (23) Tomizawa, M.; Casida, J. E. Minor Structural Changes in Nicotinoid Insecticides Confer
373 Differential Subtype Selectivity for Mammalian Nicotinic Acetylcholine Receptors. *Br. J.*
374 *Pharmacol.* **1999**, *127* (1), 115–122 DOI: 10.1038/sj.bjp.0702526.

375 (24) Tomizawa, M.; Casida, J. E. Selective Toxicity of Neonicotinoids Attributable to
376 Specificity of Insect and Mammalian Nicotinic Receptors. *Annu. Rev. Entomol.* **2003**, *48*
377 (1), 339–364 DOI: 10.1146/annurev.ento.48.091801.112731.

378 (25) Ford, K. A.; Casida, J. E. Unique and Common Metabolites of Thiamethoxam, Clothianidin,
379 and Dinotefuran in Mice. *Chem. Res. Toxicol.* **2006**, *19* (11), 1549–1556 DOI:
380 10.1021/tx0601859.

381 (26) Han, W.; Tian, Y.; Shen, X. Human Exposure to Neonicotinoid Insecticides and the
382 Evaluation of Their Potential Toxicity: An Overview. *Chemosphere* **2018**, *192* (Supplement
383 C), 59–65 DOI: https://doi.org/10.1016/j.chemosphere.2017.10.149.

384 (27) Cimino, A. M.; Boyles, A. L.; Thayer, K. A.; Perry, M. J. Effects of Neonicotinoid Pesticide
385 Exposure on Human Health: A Systematic Review. *Environ. Health Perspect.* **2017**, *125*
386 (2), 155–162 DOI: 10.1289/EHP515.

387 (28) Klarich, K. L.; Pflug, N. C.; DeWald, E. M.; Hladik, M. L.; Kolpin, D. W.; Cwiertny, D.
388 M.; LeFevre, G. H. Occurrence of Neonicotinoid Insecticides in Finished Drinking Water
389 and Fate during Drinking Water Treatment. *Environ. Sci. Technol. Lett.* **2017**, *4* (5), 168–
390 173 DOI: 10.1021/acs.estlett.7b00081.

391 (29) Richardson, S. D.; Ternes, T. A. Water Analysis: Emerging Contaminants and Current

392 Issues. *Anal. Chem.* **2018**, *90* (1), 398–428 DOI: 10.1021/acs.analchem.7b04577.

393 (30) Cwiertny, D. M.; Snyder, S. A.; Schlenk, D.; Kolodziej, E. P. Environmental Designer
394 Drugs: When Transformation May Not Eliminate Risk. *Environ. Sci. Technol.* **2014**, *48*
395 (20), 11737–11745 DOI: 10.1021/es503425w.

396 (31) Richardson, S. D.; Plewa, M. J.; Wagner, E. D.; Schoeny, R.; DeMarini, D. M. Occurrence,
397 Genotoxicity, and Carcinogenicity of Regulated and Emerging Disinfection by-Products in
398 Drinking Water: A Review and Roadmap for Research. *Mutat. Res. Mutat. Res.* **2007**, *636*
399 (1–3), 178–242 DOI: 10.1016/J.MRREV.2007.09.001.

400 (32) Schymanski, E. L.; Jeon, J.; Gulde, R.; Fenner, K.; Ruff, M.; Singer, H. P.; Hollender, J.
401 Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating
402 Confidence. *Environ. Sci. Technol.* **2014**, *48* (4), 2097–2098 DOI: 10.1021/es5002105.

403 (33) Association, A. P. H.; American Water Works Association; publication, W. E. F. *Standard*
404 *Methods for the Examination of Water and Wastewater*; 1995; Vol. 19th ed.

405 (34) Pullman, B.; Berthod, H.; Dreyfus, M. Amine-Imine Tautomerism in Adenines. *Theor.*
406 *Chim. Acta* **1969**, *15* (3), 265–268 DOI: 10.1007/BF00526203.

407 (35) Deborde, M.; von Gunten, U. Reactions of Chlorine with Inorganic and Organic
408 Compounds during Water Treatment—Kinetics and Mechanisms: A Critical Review. *Water*
409 *Res.* **2008**, *42* (1–2), 13–51 DOI: 10.1016/j.watres.2007.07.025.

410 (36) Pattison, D. I.; Davies, M. J. Absolute Rate Constants for the Reaction of Hypochlorous
411 Acid with Protein Side Chains and Peptide Bonds. *Chem. Res. Toxicol.* **2001**, *14* (10), 1453–
412 1464 DOI: 10.1021/tx0155451.

413 (37) Schwarzenbach, R.; Gschwend, P.; Imdoden, D. *Environmental Organic Chemistry*; John
414 wiley & Sons: New Jersey, 2003; Vol. Second.

415 (38) Pinkston, K. E.; Sedlak, D. L. Transformation of Aromatic Ether- and Amine-Containing
416 Pharmaceuticals during Chlorine Disinfection. *Environ. Sci. Technol.* **2004**, *38* (14), 4019–
417 4025 DOI: 10.1021/es035368l.

418 (39) Zhang, C.; Reiter, C.; Eiserich, J. P.; Boersma, B.; Parks, D. A.; Beckman, J. S.; Barnes, S.;
419 Kirk, M.; Baldus, S.; Darley-Usmar, V. M.; White, C. R. L-Arginine Chlorination Products
420 Inhibit Endothelial Nitric Oxide Production. *J. Biol. Chem.* **2001**, *276* (29), 27159–27165
421 DOI: 10.1074/jbc.M100191200.

422 (40) Karnakar, R.; Singh, S. B.; Kulshrestha, G. Persistence and Transformation of
423 Thiamethoxam, a Neonicotinoid Insecticide, in Soil of Different Agroclimatic Zones of
424 India. *Bull. Environ. Contam. Toxicol.* **2006**, *76* (3), 400–406 DOI: 10.1007/s00128-006-
425 0935-9.

426 (41) Layer, R. W. The Chemistry of Imines. *Chem. Rev.* **1963**, *63* (5), 489–510 DOI:
427 10.1021/cr60225a003.

428 (42) Smith, M. B. *March's Advanced Organic Chemistry: Reactions, Mechanisms, and*
429 *Structure*, 7th ed.; Hoboken, 2013.

(43) Nauen, R.; Ebbinghaus-Kintscher, U.; Salgado, V. L.; Kaussmann, M. Thiamethoxam Is a Neonicotinoid Precursor Converted to Clothianidin in Insects and Plants. *Pestic. Biochem. Physiol.* **2003**, *76* (2), 55–69 DOI: https://doi.org/10.1016/S0048-3575(03)00065-8.

(44) Ford, K. A.; Casida, J. E. Comparative Metabolism and Pharmacokinetics of Seven Neonicotinoid Insecticides in Spinach. *J. Agric. Food Chem.* **2008**, *56* (21), 10168–10175 DOI: 10.1021/jf8020909.

(45) Casida, J. E. Neonicotinoid Metabolism: Compounds, Substituents, Pathways, Enzymes, Organisms, and Relevance. *J. Agric. Food Chem.* **2011**, *59* (7), 2923–2931 DOI: 10.1021/jf102438c.

(46) Sultana, T.; Murray, C.; Kleywegt, S.; Metcalfe, C. D. Neonicotinoid Pesticides in Drinking Water in Agricultural Regions of Southern Ontario, Canada. *Chemosphere* **2018**, *202*, 506–513 DOI: https://doi.org/10.1016/j.chemosphere.2018.02.108.

(47) Ford, K. A.; Casida, J. E. Chloropyridinyl Neonicotinoid Insecticides: Diverse Molecular Substituents Contribute to Facile Metabolism in Mice. *Chem. Res. Toxicol.* **2006**, *19* (7), 944–951 DOI: 10.1021/tx0600696.

Chemosphere 202 (2018) 506–513

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Neonicotinoid pesticides in drinking water in agricultural regions of southern Ontario, Canada

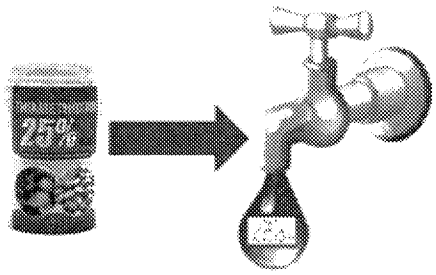
Tamanna Sultana [a], Craig Murray [b], Sonya Kleywegt [c], Chris D. Metcalfe [a, b, *]

[a] Water Quality Centre, Trent University, Peterborough, ON, Canada
[b] Institute for Watershed Science, Trent University, ON, Canada
[c] Ontario Ministry of Environment and Climate Change, Toronto, ON, Canada

HIGHLIGHTS

- Neonicotinoid insecticides detected in drinking water in rural municipalities in Canada.
- A novel method was developed for estimating concentrations from POCIS passive samplers.
- Thiamethoxam, clothianidin and imidacloprid present at the highest concentrations.
- Thiamethoxam detected in a grab sample of raw drinking water at mean concentration of 0.28 μg L^{-1}.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 December 2017
Received in revised form
16 February 2018
Accepted 17 February 2018
Available online 21 March 2018

Handling Editor: Klaus Kümmerer

Keywords:
Neonicotinoids
Drinking water
Thiamethoxam
Imidacloprid
Clothianidin
POCIS

ABSTRACT

Because of the persistence and solubility of neonicotinoid insecticides (NNIs), there is concern that these compounds may contaminate sources of drinking water. The objective of this project was to evaluate the distribution of NNIs in raw and treated drinking water from selected municipalities that draw their water from the lower Great Lakes in areas of southern Ontario, Canada where there is high intensity agriculture. Sites were monitored using Polar Organic Chemical Integrative Samplers (POCIS) and by collecting grab samples at six drinking water treatment plants. Thiamethoxam, clothianidin and imidacloprid were detected in both POCIS and grab samples of raw water. The frequency of detection of NNIs was much lower in treated drinking water, but some compounds were still detected at estimated concentrations in the low ng L^{-1} range. Thiamethoxam was detected in one grab sample of raw drinking water at a mean concentration of 0.28 μg L^{-1}, which is above the guidelines for drinking water recommended in some jurisdictions, including the European Union directive on pesticide levels <0.1 μg L^{-1} in water intended for human consumption. Further work is required to determine whether contamination of sources of drinking water with this class of insecticides is a global problem in agricultural regions.

1. Introduction

Neonicotinoid insecticides (NNIs) currently represent about one quarter of the global market for insecticides and about 80% of the market for seed treatments (Jeschke et al., 2011). Because many NNIs are relatively persistent and have high solubility in water, there is ample evidence from around the world that NNIs are

* Corresponding author. Water Quality Centre, Trent University, 1600 West Bank Drive, Peterborough, Ontario, K9J 7B8, Canada.
E-mail address: cmetcalfe@trentu.ca (C.D. Metcalfe).

https://doi.org/10.1016/j.chemosphere.2018.02.108
0045-6535/

transported from agricultural fields into surface waters (Morrissey et al., 2015; Bonmatin et al., 2015). NNIs have been widely detected in surface waters in agricultural regions of North America (Chrétien et al., 2017; Hladik and Kolpin, 2016; Hladik et al., 2014; Sanchez-Bayo and Hyne, 2014; Main et al., 2014; Starner and Goh, 2012), including in the Great Lakes basin (Hladik et al., 2018; Struger et al., 2017). There is potential for these pesticides to also contaminate sources of drinking water. For instance, three NNIs (i.e. clothianidin, imidacloprid and thiamethoxam) were detected at concentrations as high as 57 ng L^{-1} in water collected from the drinking water treatment system for the University of Iowa in the USA (Klarich et al., 2017).

NNIs are active against a wide variety of insects, but these insecticides have been reported to have relatively low acute toxicity to vertebrates (Tomizawa and Casida, 2011). However, there is evidence that chronic exposure to these compounds can induce biological effects in mammalian experimental models (Duzguner and Erdogan, 2012) and they may also affect human health (Cimino et al., 2017). Exposure to imidacloprid delayed embryonic development in mice (Gu et al., 2013) and in chick embryos (Hussein et al., 2014). Some of the transformation products of NNIs may also be toxic to vertebrates (Tomizawa, 2004; Ding and Peng, 2015).

In this study, we evaluated the concentrations of six NNIs (i.e. imidacloprid, clothianidin, thiamethoxam, acetamiprid, thiacloprid) and a hydroxy-metabolite of imidacloprid in raw (untreated) and treated drinking waters in selected municipalities in Ontario, Canada that withdraw their drinking water from the nearshore zone of the lower Great Lakes. A recent study in this agricultural region of southern Ontario showed that surface waters are highly contaminated with NNIs (Struger et al., 2017). Treated drinking water was monitored at six drinking water treatment plants (DWTPs) in July of 2015 using Polar Organic Chemical Integrative Samplers (POCIS), as well as by analysis of grab samples collected at the time of POCIS deployment and retrieval.

2. Methods and materials

2.1. Sampling

The six DWTPs in southwestern Ontario that participated in this study are described in Table 1. All DWTPs withdraw their water from the western Lake Erie watershed (i.e. western Lake Erie, Lake St. Clair, Detroit River), except for DWTP 6, which takes its water from the Grand River, ON, which is a watershed that discharges into eastern Lake Erie. Agricultural crops in these regions include corn, sorghum, soybeans, cereal grains and tobacco. The soils in the region are primarily sandy loam, and agricultural fields are extensively tile-drained (Schaafsma et al., 2015). The rated capacities of the DWTPs (Table 1) show that these facilities service relatively small municipalities in rural areas.

Two POCIS purchased from EST Labs (St. Joseph, MO, USA) were deployed at each monitoring location in the DWTPs. A single POCIS spiked with Performance Reference Compounds (PRCs) was also deployed at each location. All three samplers were placed together in a stainless-steel cage and deployed in the raw and treated water stream at each plant, with deployments in the treated drinking after disinfection by chlorination. Deployments were for approximately two weeks during July of 2015 (Table 1). Field blank POCIS spiked with PRCs were carried into the field and exposed to the air during deployment and retrieval.

The POCIS with PRCs were prepared in the laboratory by spiking 220 mg of HLB Oasis sorbent purchased from Waters (Milford, MA, USA) with 600 ng of each of the PRCs; two deuterated beta-blocker drugs, metoprolol-d_6 and propranolol-d_7, and des-isopropyl atrazine-d_5 (i.e. DIA-d_5). The spiked sorbent was placed between two polyether sulfone (PES) membranes purchased from EST Labs and assembled with standard galvanized metal rings. Data on the elimination of the PRCs from POCIS deployed in the field between deployment and retrieval were used to adjust the POCIS sampling rates to refine the estimates of time weighted average (TWA) concentrations in raw and treated water, as described below.

Grab samples (500 mL) of raw and treated drinking water (post-chlorination) were collected at the times of POCIS deployment and retrieval at all six DWTPs and were frozen for a period not exceeding 3 weeks before extraction. The samples were thawed and then aliquots (n = 3) were extracted using the SPE method described below.

2.2. Extraction

The target NNIs included imidacloprid, clothianidin, thiamethoxam, acetamiprid, thiacloprid and 5-hydroxy imidacloprid. Methods for the extraction of NNIs from grab samples of water using solid phase extraction (SPE) methods were the same as the SPE methods that we previously described for a range of pesticides (Metcalfe et al., 2016). Briefly, aliquots of water were filtered through 1.0 μm mesh glass-fiber filters and the pH was adjusted to 4.0 with H_2SO_4. After adding a mixture of stable isotope internal standards, volumes of 100 mL of water were passed through preconditioned Oasis HLB (6 cc, 200 mg) SPE cartridges purchased from Waters. After sample loading, the analytes were eluted from the SPE with 3 × 3 mL of methanol/acetone (60:40).

The procedures for extracting the target compounds from POCIS were similar to our previously described methods (Metcalfe et al., 2016). Briefly, the POCIS were dismantled and the HLB Oasis sorbent transferred to a glass column, and then internal standards were added. For POCIS that contained PRCs, acebutolol-d_5 and atrazine-d_5 were also added as internal standards prior to extraction to aid in the analysis of the PRCs. The target analytes were eluted from the POCIS sorbent in the column with a volume of 200 mL of methanol. All extracts were evaporated to near-dryness and reconstituted in 0.4 mL of methanol. Procedural blanks were extracted concurrently with water samples and with POCIS.

Table 1
Drinking water treatment plants (DWTPs) monitored in Ontario, Canada for neonicotinoid pesticides, and the dates of POCIS deployment and the mean temperatures and pH in the raw and treated drinking water.

Plant	Source of drinking water	Rated Flow Capacity (L sec^{-1})	POCIS Deployment Date	POCIS Retrieval Date	Days Deployed	Mean Raw pH	Mean Raw Temp (°C)	Mean Treated pH	Mean Treated Temp (°C)
DWTP 1	Lake Erie	118.4	2-Jul-2015	17-Jul-2015	15	7.9	22.2	7.2	22.4
DWTP 2	Detroit River	210.5	2-Jul-2015	17-Jul-2015	15	7.95	21.7	7.32	21.2
DWTP 3	Lake St. Clair	421.3	3-Jul-2015	17-Jul-2015	14	7.98	21.1	7.48	21.1
DWTP 4	Lake St. Clair	52.6	3-Jul-2015	17-Jul-2015	14	7.73	21.7	7.34	22.1
DWTP 5	Lake Erie	1442	3-Jul-2015	17-Jul-2015	14	8.3	21.8	7.6	22.3
DWTP 6	Grand River	46.9	2-Jul-2015	15-Jul-2015	13	8.15	23.6	7.25	23.2

2.3. Analysis

Analysis of target compounds and their stable isotope surrogates was by liquid chromatography and tandem mass spectrometry with electrospray ionization (LC-ESI-MS/MS) using a QTrap 5500 instrument coupled with an Agilent 1100 HPLC purchased from Applied Biosystems-Sciex (Concord, ON, Canada). The injection volume was 20 μL. Analytes were separated chromatographically using a Genesis Reverse Phase C18 (150 × 2.1 mm ID, 4 μm particle size) column coupled to a guard column with the same packing material (4 mm × 2.0 mm); both supplied by Phenomenex (Torrance, CA, USA). The column was not heated and it was eluted with a binary mixture of 2 mM ammonium acetate in MilliQ water with 0.1% acetic acid (Solvent A) and acetonitrile with 0.1% acetic acid (Solvent B). The solvent gradient was: start at 10% Solvent B in Solvent A, then increase to 50% by 3 min and continue increasing to 97% Solvent B by 5 min and then hold for a minute at 97% Solvent B. Continue the increase in the proportion of Solvent B to 98% by 8 min, 99% by 10 min and then hold for a minute. By 12 min, reduce the Solvent B gradient back to 10% Solvent B and hold for 7 min, for a total run time of 19 min. The mobile phase flow was 340 μL min^{-1}.

MS detection was performed using multiple reaction monitoring (MRM) in positive ion mode. The ionization conditions were: Ion spray voltage of 4500 V, temperature of 550 °C, pressure of the ion source gases of 35 psi. The collision gas was helium, set at high flow. The ion transitions monitored for each target compound, the internal standards and the PRCs, as well as the suppliers of these compounds are listed in Supplemental Information (Table S1). Quantification of all compounds was performed using an external standard method with a nine-point calibration curve from 1 to 200 ng mL^{-1} generated for each analyte. The response to the internal standard for each target compound (50 ng mL^{-1}) was used to account for matrix effects and recoveries.

Procedural blanks were processed as described for the field samples, but the target analytes were not detected in any of these blank samples. The instrumental detection limits (IDL) were defined as the lowest amount of analyte (ng) that produced a peak with a signal-to-noise ratio of 3. The Limits of Detection (LODs) for grab samples were calculated from the IDL (ng) divided by the sample volume (100 mL). The LODs for POCIS were calculated from the IDL (ng) divided by the total volume of water sampled over the 14 d deployment. The LODs of the target analytes ranged from 1 to 6 ng L^{-1} for grab samples and 0.1–2.1 ng L^{-1} for POCIS (Table S1). The LODs for the NNI that were detected are also listed in Tables 3 and 4.

2.4. POCIS sampling rates

POCIS sampling rates were determined using a static, daily renewal protocol with the NNIs spiked into a synthetic "US EPA Medium Hard Water", which has a pH of ~7.5, hardness of 80 mg L^{-1} and alkalinity of 60 mg L^{-1} (US EPA, 2002). The recipe for preparation of this synthetic water and the protocols for determining the sampling rates are described in detail in Supplementary Information (Table S2). Briefly, single POCIS were placed in a vertical orientation in 3 L of test medium spiked with the NNI compounds. The spiked test medium in each of the test containers was replaced every day. The tests were conducted for 14 d in an environmental chamber at a temperature of 15 °C and in the dark. Every day over the 14 d test period, a sample of water (10 mL) was removed from three randomly selected test containers and the control container to monitor the concentrations of the NNIs in the water. On each of days 0, 5, 10 and 14, three POCIS were collected for analysis of the amounts of the target compounds accumulated in the samplers.

2.5. PRC elimination rates

The rates of elimination of PRCs from spiked POCIS were determined in the laboratory using a static, daily renewal protocol with a matrix of US EPA moderately hard water. POCIS spiked with DIA-d_5, propranolol-d_7 and metoprolol-d_5 (i.e. PRC-POCIS) were placed in 3 L of test medium in each test container in a vertical orientation. A "control" container contained 3 L of the test medium, but no PRC-POCIS. The tests were conducted for 14 d in an environmental chamber at a temperature of 15 °C and in the dark. The test matrix in each test container was stirred with a magnetic stirrer at approximately 75 rpm. The test medium was replaced every day in each of the test containers. In addition, every day over the 14 d test period, a sample of water (10 mL) was removed from three randomly selected test containers and the control container to monitor the concentrations of PRCs in the water. On each of days 0, 5, 10 and 14, three PRC-POCIS were collected to monitor losses of the PRCs from the POCIS over time.

2.6. Estimating TWA concentrations

The theory for determining sampling rates for POCIS was previously described in detail by Sultana et al. (2017), and a description of the methods used to estimate the time weighted average (TWA) concentrations of the target compounds is included in Supplemental Information. Experimentally determined sampling rates (Rs_{cal}) were adjusted according to the rates of elimination of PRCs from POCIS deployed in the field to calculate a field sampling rate (Rs_{field}). The TWA concentrations of individual NNI analytes in water (C_W) were then calculated from the mass (ng) accumulated on the POCIS (m_s) over the deployment period in days (t) according to:

$$C_W = m_s / \left(Rs_{field} \times t \right) \quad (1)$$

3. Results and discussion

3.1. POCIS sampling rates

Using a static, daily renewal protocol with synthetic water over 14 d, sampling rates (Rs_{cal}) were estimated for the target compounds (Table 2). Note that it was necessary to renew test solutions every day and with a single POCIS in each 3 L test container to ensure that the concentrations of the target compounds were maintained at levels within 10% of the nominal concentrations. Monitoring of the test compounds in the spiked medium confirmed that concentrations of NNIs were within ±10% of the nominal concentrations.

Whether a passive sampler behaves as a kinetic or equilibrium sampler is highly dependent on the partitioning properties of the target chemical, the concentration in the water and the length of the deployment period (Vrana et al., 2005). As illustrated in Fig. 1 for imidacloprid, the uptake profiles of all NNIs were linear over 14 d, indicating that the POCIS is a kinetic sampler for NNIs over this deployment period, provided the amounts sequestered on the sorbent do not exceed the maxima observed in the calibration experiment.

From the slopes for accumulation of the target analytes and the data on the average concentrations of the compounds in water over the test period, the POCIS sampling rates were estimated (Table 2). Except for thiacloprid and dinotefuran, all sampling rates are within the range of 0.1–0.5 L d^{-1} that are typical for POCIS (Harman et al., 2012). The sampling rates for NNIs estimated by Ahrens et al. (2015)

Table 2
Chemical properties and sampling rates (Rs_{cal}) of the target NNIs. Database source: http://sitem.herts.ac.uk/aeru/iupac/.

Chemical	MW g mol^{-1}	Log Kow at 20 °C	Solubility mg L^{-1}	pKa	Rs_{cal} L d^{-1}
Imidacloprid	255.7	0.57	610	1.56	0.18
Imidacloprid-OH	271.7	–	–	–	0.14
Thiamethoxam	291.7	−0.13	4100	0.41	0.10
Clothianidin	249.7	0.91	340	11.09	0.19
Thiacloprid	252.7	1.26	185	1.03	0.06
Acetamiprid	222.7	0.80	2950	0.7	0.17
Dinotefuran	202.2	−0.55	54,300	12.6	0.02

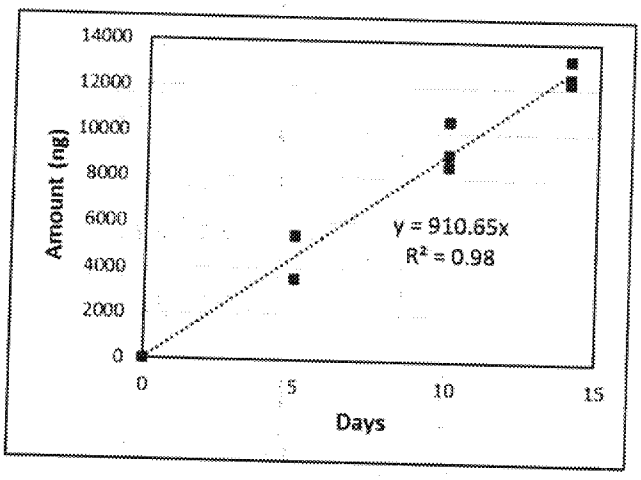


Fig. 1. Amounts (ng) of imidacloprid adsorbed to POCIS sorbent (n = 3) on Days 0, 5, 10 and 14 in a static, daily renewal protocol with US EPA moderately hard water at 15 °C.

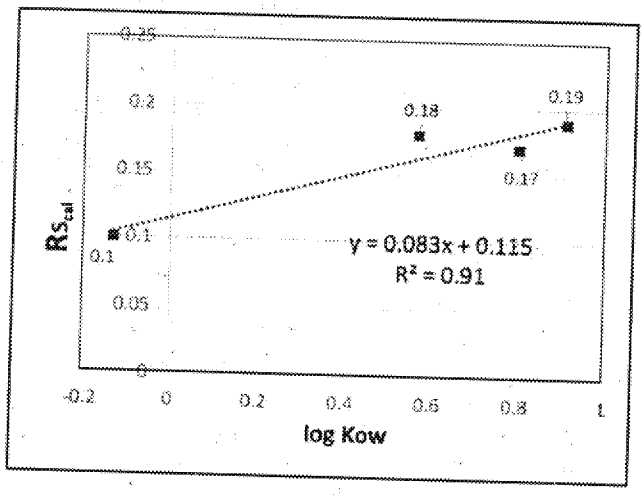


Fig. 2. Relationship between log Kow and POCIS sampling rate (Rs_{cal}) for imidacloprid, thiamethoxam, clothianidin and acetamiprid.

in a static, non-renewal test with river water for imidacloprid (i.e. 0.18), thiamethoxam (i.e. 0.11) and clothianidin (i.e. 0.21) were in good agreement with the Rs_{cal} values determined in the present study.

Sánchez-Bayo and Hyne (2014) developed a passive sampling method for monitoring NNIs using sorbent placed in Empore™ disks and found that sampling rates were positively related to hydrophobicity. We found a positive relationship between log Kow and the sampling rates ($r^2 = 0.91$) for clothianidin, imidacloprid, acetamiprid and thiamethoxam (Fig. 2), but this relationship did not hold if the most hydrophobic (i.e. thiacloprid) and the least hydrophobic (i.e. dinetofuran) compounds were included.

Note that two of the NNIs are weak bases (i.e. clothianidin, dinotefuran) and all others are weak acids (Table 2), so these compounds are likely to be present as anions or cations, respectively at the pH of natural waters, which may influence adsorption to the HLB Oasis mixed-phase sorbent in the POCIS (Li et al., 2011). The US EPA moderately hard water test matrix was selected to approximate the pH, alkalinity and hardness of surface waters in the study region, but this matrix does not replicate all the chemical properties of these waters. This synthetic water was originally developed as a standard matrix for toxicity testing, and there are other formulations that can be used to replicate harder or softer water (US EPA, 2002). Previous studies to determine the sampling rates for contaminants using POCIS and other passive samplers have been conducted with a variety of aqueous matrixes that vary in pH, ionic strength, etc. This novel approach of conducting tests with an accepted synthetic matrix may be a step towards developing a standardized approach for determining sampling rates for passive samplers.

3.2. PRC elimination

The test to determine losses over time of the PRCs (i.e. DIA-d_5, propranolol-d_7 and metoprolol-d_6) from spiked POCIS were also conducted in the laboratory with US EPA moderately hard water (daily renewal) over 14 d at 15 °C. The elimination rate constants (i.e. $K_{e\ PRC\text{-}cal}$) were estimated from the first order kinetics of losses of the PRCs over time. Elimination rates per day were very consistent for the three PRCs, at −0.0249 for metoprolol-d_6, -0.0266 for propranolol-d_7 and -0.0245 for DIA-d_5. We previously evaluated the use of propranolol-d_7 and metoprolol-d_6 as PRCs in POCIS and concluded that these basic compounds were suitable because they showed elimination between 50 and 80% over 10 d in a flow-through system (Sultana et al., 2017). The acidic compound, DIA-d_5 has been previously used as a PRC for monitoring pesticides in surface waters (Lissalde et al., 2014). Mazzella et al. (2010) reported a higher rate of elimination for DIA-d_5 of −0.057, but these authors used very different experimental conditions for determining the rates of elimination of this compound. The rates of elimination of these PRCs with different chemical properties were used as an indicator of the flux of target NNIs between the aquatic environment and the POCIS sorbent. The use of different PRCs to adjust the POCIS sampling rates for acidic and basic compounds is a novel approach that makes a significant contribution to the development of POCIS as a monitoring tool for pesticides and other water-soluble contaminants.

All PRC-spiked POCIS deployed in the field were analyzed for the amounts of the three PRCs remaining in the samplers at the end of the deployment period. The data on the amounts of these compounds in the POCIS after deployment relative to the amounts in

the field blanks were used to estimate elimination rates (i.e. $K_{e\ PRC\text{-}field}$). The ratio between elimination rates in the field and in the laboratory were used to calculate Exposure Adjustment Factors (EAF). The EAFs calculated from PRC data for POCIS deployed at the six DWTPs are summarized in Supplemental Information (Table S3). EAF values > 1 indicate that the PRCs were eliminated more rapidly from POCIS deployed in the field relative to the elimination of the PRC compounds observed in the laboratory. A variety of *in situ* environmental factors can increase the rates of flux across the POCIS membrane, including higher water temperatures and flows (Harman et al., 2012). The rates of elimination of PRCs were determined in the laboratory at 15 °C and with only light mixing, while there were high flows and water temperatures in the range of 21–24 °C in the drinking water treatment plants (Table 1). Therefore, the EAF values > 1 observed in most cases were expected. Biofouling can reduce the flux of compounds in POCIS (Harman et al., 2012) and some biofouling was observed in field deployed POCIS; especially for those devices deployed in the raw water. This may explain the relatively low EAF values calculated from PRC-POCIS deployed in raw water in DWTP1, DWTP4 and DWTP6 (Supplemental Information, Table S3). The pH of raw drinking water (Table 1) was slightly higher than the pH ~7.5 of the medium used to determine PRC elimination in the laboratory, so this may also have altered the flux of PRCs in the field.

There was generally good agreement between EAF values calculated from all three PRCs, except in DWTP 4 and DWTP 6, where there were differences between EAFs for the two basic PRCs (i.e. metoprolol-d_6, propranolol-d_7) and the EAF for DIA-d_5. Therefore, the EAF values determined from elimination rates for DIA-d_5 were applied to all acidic NNIs and the average of the two EAF values for metoprolol-d_6 and propranolol-d_7 were applied to the only basic NNI that was detected, clothianidin. Unfortunately, no PRC data were available for POCIS deployed in treated water at DWTP 3, so the average EAF values calculated for the PRC-POCIS deployed in treated water across the other five DWTPs was applied to calculating the sampling rates of NNIs in treated water at DWTP3.

3.3. NNIs in drinking water

The data on the mean concentrations from replicate (n = 3) analysis of the NNIs in grab samples of raw and treated drinking water collected on the day of POCIS deployment (i.e. Sample 1) and approximately two weeks later at the day of retrieval (i.e. Sample 2) are summarized in Table 3. Thiamethoxam and imidacloprid were detected in both raw and treated drinking water, and clothianidin was detected in only raw water (Table 3). However, the data for grab samples were highly variable. For instance, relatively high mean concentrations of thiamethoxam were detected in treated drinking water from DWTP 4 in Sample 1, but this compound was not detected at all in treated water from this location in Sample 2 (Table 3). Conversely, imidacloprid was detected in Sample 2, but not in Sample 1 (Table 3). The mean concentrations of the analytes in grab samples of treated drinking water were always lower than in the raw water. Acetamiprid, dinotefuran and the hydroxy-transformation product of imidacloprid were not detected at concentrations above the LOD, and thiacloprid was only detected in raw water from DWTP 6 (Table 3)). Overall, the ranking of the mean measured concentrations of the NNIs detected in grab samples of raw drinking water were thiamethoxam > clothiamidin > imidacloprid > thiacloprid.

The data on the amounts of the NNIs (m_s) accumulated on the POCIS by the end of the deployment period are summarized in Supplemental Information (Table S4), and the PRC-corrected

Table 3
Mean (±SD in brackets) concentrations of NNIs (ng L^{-1}) in replicate analyses (n = 3) of grab samples of raw and treated drinking water collected on two separate days from each of six drinking water treatment plants located in Ontario, Canada. ND = not detected at concentrations > LOD.

	Thiamethoxam				Clothianidin				Thiacloprid				Imidacloprid				Acetamiprid			
LODs	1 ng L^{-1}				4 ng L^{-1}				1 ng L^{-1}				2 ng L^{-1}				3 ng L^{-1}			
SITE	Sample 1		Sample 2		Sample 1		Sample 2		Sample 1		Sample 2		Sample 1		Sample 2		Sample 1		Sample 2	
	Raw	Treated	Raw	Treated	Raw	Treated	Raw	Treated	Raw	Treated	Raw	Treated	Raw	Treated	Raw	Treated	Raw	Treated	Raw	Treated
DWTP 1	32.2 (±2.2)	ND	ND	ND	ND	ND	7.2 (±1.1)	ND	ND	ND	ND	ND	ND	ND	2.7 (±1.2)	ND	ND	ND	ND	ND
DWTP 2	52.7 (±7.0)	32.5 (±5.2)	ND	NG	33.2 (±4.1)	ND	6.8 ±2.5)	ND	ND	ND	ND	ND	ND	ND	4.4 (±1.1)	4.8 (±0.6)	ND	ND	ND	ND
DWTP 3	79.2 (±18.3)	ND	10.8 (±1.4)	ND	57.1 (±4.3)	ND	28.7 (±7.7)	ND	ND	ND	ND	ND	ND	ND	2.7 (±1.2)	ND	ND	ND	ND	ND
DWTP 4	283.5 (±9.6)	91.7 (±6.0)	23.4 (±3.4)	ND	86.9 (±20.6)	ND	48.2 (±7.6)	ND	ND	ND	ND	ND	ND	ND	8.6 (±1.0)	ND	ND	ND	ND	ND
DWTP 5	38.9 (±2.5)	20.1 (±2.2)	ND	ND	ND	ND	5.9 (±1.3)	ND	ND	ND	ND	ND	ND	ND	4.3 (±0.2)	2.4 (±2.2)	ND	ND	ND	ND
DWTP 6	42.9 (±1.1)	30.0 (±2.2)	18.2 (±2.7)	ND	138.1 (±10.6)	ND	77.1 (±7.6)	ND	ND	ND	2.7 (±1.0)	ND	ND	ND	13.5 (±1.4)	4.7 (±1.3)	ND	ND	ND	ND

sampling rates (Rs_{field}) for both sides of each treatment plant are also summarized in Supplemental Information (Table S5). The TWA concentrations (ng L^{-1}) summarized in Table 4 were estimated from these data according to Equation (1).

Overall, the TWA concentrations estimated from the POCIS were consistent with the data generated from grab samples. Both data sets show that the lowest concentrations of the NNIs in raw drinking water were observed in DWTP 1 and DWTP 5, while the highest were from DWTP 4 and DWTP 6. The data from both the grab samples (Table 3) and POCIS (Table 4) indicate that thiamethoxam was present at higher concentrations than clothianidin in all DWTPs, except at DWTP 4, where the estimated TWA concentrations of these two NNIs were approximately equal (Table 4). Although imidacloprid was detected frequently in raw water, the transformation product of this compound (i.e. 5 hydroxy-imidacloprid) was not detected in any of the samples. This transformation product was included among the target analytes because imidacloprid is known to be present at high concentrations in surface waters (Morrissey et al., 2015) and also, we were able to obtain an analytical standard for this compound. Dinotefuran was also not detected in any of the grab samples or in the POCIS. Acetamiprid was detected at low TWA concentrations estimated from the POCIS samples (Table 4), but not in the grab samples (Table 3).

The POCIS that were spiked with PRCs in the laboratory (i.e. Replicate 3) generated estimates of concentrations of NNIs that were often slightly lower than the POCIS purchased directly from the supplier (Table 4). This may reflect differences in the methods for fabricating the samplers from the commercial suppliers and in the laboratory, respectively. The frequency of detection of the target compounds in POCIS deployed in treated drinking water was much higher than the frequency of detection in grab samples. There is also a very high frequency of non-detects of NNIs in the current monitoring program for treated drinking water by the Ontario Ministry of Environment and Climate Change. This illustrates the value of using passive samplers for routine monitoring to concentrate the target compounds to detectable levels, as was also shown in our previous survey of pharmaceuticals in drinking water using POCIS (Metcalfe et al., 2014).

In general, the results of this study are consistent with the previously published study on the levels of NNIs in the drinking water treatment and distribution system for the University of Iowa, USA, where three insecticides at relative concentrations of clothianidin > imidacloprid > thiamethoxam were detected in grab samples collected in June and July of 2016, with concentrations of clothianidin as high as 57.3 ng L^{-1} (Klarich et al., 2017). This maximum concentration is slightly lower than the maximum concentrations of clothianidin measured in grab samples of raw drinking water from DWTP 6 (Sample 1), but it is consistent with the TWA concentrations estimated at this site from *in situ* POCIS monitoring. However, the mean concentration of thiamethoxam observed in one of the grab samples of raw water from DWTP 4 (i.e. Sample 1) was 283.5 ng L^{-1}. DWTP 4 takes its water from an intake in Lake St. Clair that is highly impacted by agricultural runoff that transported into the Thames River watershed in Ontario (Li et al., 2003). NNIs have recently been shown to be present at high frequencies in the Thames River (Struger et al., 2017). Fig. 3a illustrates the daily discharge measured in the Thames River over the monitoring period in 2015, and these data show that there was a large precipitation-driven increase in river discharge just before the POCIS were deployed at DWTP 4 that may have contributed to the transport of NNIs into the source of drinking water. Similarly, there were precipitation-driven increases in the discharge of the Grand River over the period of POCIS deployment (Fig. 3b), and this may have contributed to the transport of the NNIs that were detected in DWTP 6. The high proportion of tile-drained agricultural fields in the region would have contributed to the rapid transport of NNIs into sources of drinking water (Chrétien et al., 2017).

However, we may have missed the peak concentrations of NNIs in drinking water because the POCIS were deployed and the first grab samples collected at the beginning of July. NNIs are typically applied to agricultural fields in Ontario, Canada in early June, so an

Table 4
Estimated TWA concentrations of NNIs (ng L^{-1}) in replicate POCIS ($n = 3$) deployed in raw and treated drinking water at each of six drinking water treatment plants (DWTPs) in Ontario, Canada and in field blank POCIS. ND = not detected at concentrations > LOD.

Estimated LODs	Thiamethoxam 0.2 ng L^{-1}		Clothianidin 0.2 ng L^{-1}		Thiacloprid 0.2 ng L^{-1}		Imidacloprid 0.1 ng L^{-1}		Acetamiprid 0.1 ng L^{-1}	
	Raw	Treated	Raw	Treated	Raw	Treated	Raw	Treated	Raw	Treated
Field Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DWTP 1 Rep 1	0.36	0.24	0.92	ND	0.33	ND	0.20	ND	0.13	ND
DWTP 1 Rep 2	0.54	0.24	0.69	0.20	0.30	ND	ND	ND	0.11	ND
DWTP 1 Rep 3	0.28	ND	0.29	0.21	0.12	ND	1.96	ND	0.10	ND
Field Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DWTP 2 Rep 1	8.04	7.55	0.62	0.51	0.21	0.10	0.25	ND	0.61	0.10
DWTP 2 Rep 2	11.89	7.37	0.92	0.57	0.17	ND	0.34	0.25	0.82	0.17
DWTP 2 Rep 3	4.78	4.94	0.90	0.64	ND	ND	0.50	0.24	0.30	ND
Field Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DWTP 3 Rep 1	8.39	0.25	7.51	ND	0.17	0.15	1.30	ND	0.57	0.10
DWTP 3 Rep 2	8.53	ND	8.64	0.20	0.14	0.15	1.44	ND	0.42	0.10
DWTP 3 Rep 3	5.15	0.24	5.18	0.22	ND	0.19	0.54	ND	0.10	ND
Field Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DWTP 4 Rep 1	59.47	5.42	43.65	0.35	0.34	ND	3.18	0.19	1.10	0.21
DWTP 4 Rep 2	62.58	5.70	41.04	0.54	0.35	0.17	2.23	0.10	1.31	0.23
DWTP 4 Rep 3	48.91	4.17	33.19	0.29	0.42	ND	1.22	0.18	0.16	ND
Field Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DWTP 5 Rep 1	2.38	0.44	0.27	0.24	0.20	0.14	1.11	ND	0.19	0.14
DWTP 5 Rep 2	1.41	0.65	0.53	0.33	0.20	0.13	0.28	ND	0.19	0.12
DWTP 5 Rep 3	1.44	0.28	0.24	ND	ND	ND	1.14	0.12	0.10	ND
Field Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DWTP 6 Rep 1	17.73	0.26	5.72	0.25	0.27	ND	1.29	0.76	1.24	0.27
DWTP 6 Rep 2	11.14	0.35	6.75	0.45	0.25	ND	0.95	0.15	0.94	0.51
DWTP 6 Rep 3	8.75	0.31	5.57	0.23	0.20	ND	0.94	0.10	0.26	0.11

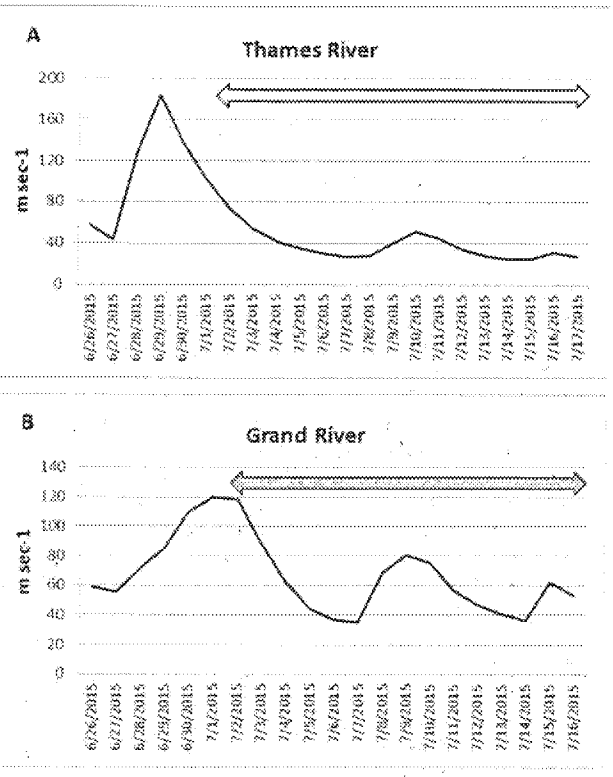


Fig. 3. Mean daily discharge (m^3 sec^{-1}) in the Thames River (A) and the Grand River (B) over the period from June 26 to July 17, 2015. The period of POCIS deployment is shown by the double-arrowed line. The discharge data were extracted from the national data base maintained by Water Survey of Canada for the hydrometric stations located at Thamesville on the Thames River and (Station 02GE005) and at Brantford on the Grand River (Station 02GB001); https://wateroffice.ec.gc.ca.

earlier deployment period may have revealed higher concentrations of these compounds in drinking water. This may explain why the concentrations in grab sample 1 were often elevated relative to the concentrations in grab sample 2 or the TWA concentrations estimated over the period of deployment of the POCIS.

In the drinking water study in Iowa, USA, there appeared to be little removal of the NNIs throughout the treatment train (Klarich et al., 2017). In the present study, the POCIS data showed that the concentrations of NNIs were generally lower in treated drinking water relative to raw water, with the exception of DWTP 2. All municipalities included in this survey treat their drinking water using conventional treatment systems, including coagulation, flocculation, sedimentation, filtration and chlorine disinfection, so it is not clear why NNIs were present at higher concentrations in treated drinking water in some municipalities relative to others. Since all DWTPs included in the present study disinfect by chlorination, it would be especially important to evaluate whether there are chlorination bi-products of NNIs present in drinking water. Bi-products of chlorination have been identified for several pesticides (e.g. Xu et al., 2017; Ibáñez et al., 2011), but to our knowledge, no studies have been done to determine if bi-products are formed by chlorination of residues of NNIs in drinking water. Because NNIs were detected in tap water monitored in the study in Iowa, USA (Klarich et al., 2017), more work is needed to determine whether NNIs and transformation products persist in the tap water for Ontario municipalities.

Overall, these data indicate that NNIs can contaminate sources of drinking water and that conventional water treatment technologies are not completely effective at removing these compounds. The concentrations of these insecticides in drinking water (ng L^{-1}) were orders of magnitude lower than the current Water Quality Guidelines for Drinking Water established by Health Canada for clothianidin, imidaclopid and thiamethoxam of 300, 500 and 5 μg L^{-1}, respectively. For comparison, the mean concentration of thiamethoxam detected in one grab sample from DWTP 4 was 0.28 μg L^{-1}. On the other hand, the European Union directive on pesticide levels in water intended for human consumption, addressed in the Drinking Water Directive and the Groundwater Directive states that concentrations of pesticides may not exceed 0.1 μg L^{-1} for a single pesticide and 0.5 μg L^{-1} for total pesticides, so the levels of NNIs in raw drinking water in the present study exceeded those thresholds. There is evidence that there is potential for human health effects from exposure to NNIs or their transformation products (Cimino et al., 2017; Ding and Peng, 2015). Therefore, further studies are needed to evaluate health risks to human populations chronically exposed to these compounds or their transformation products. In a study conducted in China among populations that drink surface water and well water in regions with different intensities of pesticide use for rice cultivation, Lai (2017) observed a positive relationship between the degree of pesticide use and scores for a medical disability index among individuals >65 years of age. Similar approaches could be used with data on neonicotinoid use in the Great Lakes basin and other regions to investigate whether exposure to this class of insecticides in drinking water is associated with negative health outcomes.

The present study contributes to the literature that shows that pesticides are contaminants of drinking water. Along with the previous study by Klarich et al. (2017), the present study demonstrates that NNIs can be transported into sources of drinking water in agricultural regions where there is heavy use of this class of insecticides. The present study advances our understanding of the scope of NNI contamination by showing that residues were present in drinking water in 6 different DWTPs serving rural communities in the Great Lakes basin. More work is required to determine whether contamination of drinking water by NNIs is a widespread problem in other agricultural regions of the globe.

Acknowledgements

This project received funding support from the Government of Ontario. Such support does not indicate endorsement by the Government of Ontario of the contents of this contribution. We thank Brenda Seaborn for preparing the samples for analysis. The cooperation of the municipal offices and staff at the drinking water treatment plants is greatly appreciated.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.02.108.

References

Alvarez, L., Daneshvar, A., Lau, A.E., Kreuger, J., 2015. Characterization of five passive sampling devices for monitoring pesticides in water. J. Chromatogr. A 1405, 1–11.

Bonmatin, J.M.M., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C., Liess, M., Long, E., Marzaro, M., Mitchell, E.A.D., Noome, D., Simon-Delso, N., Tapparo, A., 2015. Environmental fate and exposure; neonicotinoids and fipronil. Environ. Sci. Pollut. Res. 22, 5–62.

Chrétien, E., Giroux, I., Theriault, G., Gagnon, P., Corriveau, J., 2017. Surface runoff and subsurface tile drain losses of neonicotinoids and companion herbicides at edge-of-field. Environ. Pollut. 224, 255–264.

Cimino, A.M., Boyles, A.L., Thayer, K.A., Perry, M.J., 2017. Effects of neonicotinoid pesticide exposure on human health: a systematic review. Environ. Health Persp. 125, 155–162.

Ding, F., Peng, W., 2015. Biological assessment of neonicotinoids imidacloprid and its major metabolites for potentially human health using globular proteins as a model. J. Photochem. Photobiol. B Biol. 147, 24–36.
Duzguner, V., Erdogan, S., 2012. Chronic exposure to imidacloprid induces inflammation and oxidative stress in the liver and central nervous system of rats. Pestic. Biochem. Physiol. 104, 58–64.
Gu, Y., Li, K., Hang, X., Zheng, J., Yang, J., Diao, N., Yuan, Y., Ke, Y., Liu, M., Shi, H., Xu, W., 2013. Reproductive effects of two neonicotinoid insecticides on mouse sperm function and early embryonic development in vitro. PLoS One 8, e70112.
Harman, C., Allan, I.J., Vermeirssen, E.L.M., 2012. Calibration and use of the polar organic chemical integrative sampler – a critical review. Environ. Toxicol. Chem. 31, 2724–2738.
Hladik, M.L., Corsi, S.R., Kolpin, D.W., Baldwin, A.K., Blackwell, B.R., Cavallin, J.E., 2018. 2018 Year-round presence of neonicotinoid insecticides in tributaries to the Great Lakes, USA. Environ. Pollut. 239, 1022–1029.
Hladik, M.L., Kolpin, D.W., 2016. First national-scale reconnaissance of neonicotinoid insecticides in streams across the USA. Environ. Chem. 13, 12–20.
Hladik, M.L., Kolpin, D.W., Kuivila, K.M., 2014. Widespread occurrence of neonicotinoid insecticides in streams in a high corn and soybean producing region, USA. Environ. Pollut. 193, 189–196.
Hussein, M., Singh, V., Hassan, M.A., Singh, A.R., Yadav, B., 2014. Malformations and teratogenic effects of imidacloprid on chick embryo. Scholars J. Appl. Med. Sci. 2, 67–72.
Ibáñez, M., Sancho, J.V., Pozo, J., Hernández, F., 2011. Use of quadrupole time-of-flight mass spectrometry to determine proposed structures of transformation products of bromoxil after water chlorination. Rapid Commun. Mass Spectrom. 24, 3103–3113.
Jeschke, P., Nauen, R., Schindler, M., Elbert, A., 2011. Overview of the status and global strategy for neonicotinoids. J. Agric. Food Chem. 59, 2897–2908.
Klarich, K.L., Pflug, N.C., DeWald, E.M., Hladik, M.L., Kolpin, D.W., Cwiertny, D.M., LeFevre, G.H., 2017. Occurrence of neonicotinoid insecticides in finished drinking water and fate during drinking water treatment. Environ. Sci. Technol. Lett. 4, 168–173.
Lai, W., 2017. Pesticide use and health outcomes: evidence from agricultural water pollution in China. J. Environ. Econ. Manag. 86, 93–120.
Li, H., Helm, P.A., Paterson, G., Metcalfe, C.D., 2011. The effects of dissolved organic matter and pH on sampling rates for polar organic chemical integrative samplers (POCIS). Chemosphere 83, 271–280.
Li, Y., Li, Y.F., Struger, J., Chen, B., Huang, G.H., 2007. Pesticide runoff model (PeRM): an integrated modeling system for simulation of pesticide runoff losses from agricultural lands. J. Environ. Sci. Health B 38, 257–273.
Lissalde, S., Mazzella, N., Mazellier, P., 2014. Polar organic chemical integrative samplers for pesticide monitoring: impacts of field exposure conditions. Sci. Total Environ. 468–469, 188–196.
Main, A.R., Headley, J.V., Peru, K.M., Michel, N.L., Cessna, A.J., Morrissey, C., 2014. Widespread use and frequent detection of neonicotinoid insecticides in wetlands of Canada's prairie pothole region. PLoS One 9, e92821.
Mazzella, N., Lissalde, S., Moreira, S., Delmas, F., Mazellier, P., Huckins, J.N., 2010. Evaluation of the use of performance reference compounds in an Oasis-HLB adsorbent based passive sampler for improving water concentration estimates of polar herbicides in freshwater. Environ. Sci. Technol. 44, 1713–1719.
Metcalfe, C.D., Sultana, T., Li, H., Helm, P.A., 2016. Fungicides and current-use herbicides in urban receiving waters in Ontario, Canada monitored using POCIS passive samplers. J. Great Lakes Res. 42, 1472–1482.
Metcalfe, C.D., Roque, M.E., Sultana, T., Murray, C., Helm, P., Kleywegt, S., 2014. Monitoring for contaminants of emerging concern in drinking water using POCIS passive samplers. Environ. Sci. Process. Impact. 16, 77–481.
Morrissey, C.A., Mineau, P., Devries, J.H., Sanchez-Bayo, F., Liess, M., Cavallaro, M.C., Liber, K., 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: a review. Environ. Int. 74, 291–303.
Sánchez-Bayo, F., Hyne, R.V., 2014. Detection and analysis of neonicotinoids in river waters – development of a passive sampler for three commonly used insecticides. Chemosphere 99, 143–151.
Schaafsma, A., Limay-Rios, V., Baute, T., Smith, J., Xue, Y., 2015. Neonicotinoid insecticide residues in surface water and soil associated with commercial maize (corn) fields in southwestern Ontario. PLoS One 10, e118139.
Starner, K., Goh, K.S., 2012. Detections of the neonicotinoid insecticide imidacloprid in surface waters of three agricultural regions of California, USA, 2010–2011. Bull. Environ. Contam. Toxicol. 88, 316–321.
Struger, J., Grabuski, J., Cagampan, S., McGoldrick, D., Marvin, C.H., 2017. Factors influencing the occurrence and distribution of neonicotinoid insecticides in surface waters of southern Ontario, Canada. Chemosphere 169, 516–523.
Sultana, T., Murray, C., Roque, M.E., Metcalfe, C.D., 2017. Monitoring contaminants of emerging concern from tertiary treatment plants using passive sampling modelled with performance reference compounds. Environ. Monit. Assess. 189, 1–10.
Tomizawa, M., Casida, J.E., 2011. Neonicotinoid insecticide toxicology: mechanisms of selective action. Annu. Rev. Pharmacol. Toxicol. 45, 247–268.
Tomizawa, M., 2004. Neonicotinoids and derivatives: effects in mammalian cells and mice. J. Pestic. Sci. 29, 177–183.
US EPA, 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, fourth ed. US Environmental Protection Agency, Washington, DC, USA, p. 335. EPA-821-R-02-013.
Vrana, B., Mills, G.A., Allan, I.J., Dominiak, E., Svensson, K., Knutsson, J., Morrison, G., Greenwood, R., 2005. Passive sampling techniques for monitoring pollutants in water. Trends Anal. Chem. 24, 845–868.
Xu, X., Wang, D., Li, C., Peng, H., Wang, Z., 2017. Characterization of the reactivity and chlorinated products of carbazole during aqueous chlorination. Environ. Pollut. 225, 412–418.

SCIENTIFIC REPORTS

OPEN

Effects of Neonicotinoid Insecticides on Physiology and Reproductive Characteristics of Captive Female and Fawn White-tailed Deer

Received: 19 September 2018
Accepted: 22 February 2019
Published online: 14 March 2019

Elise Hughes Berheim[1], Jonathan A. Jenks[1], Jonathan G. Lundgren[2], Eric S. Michel[1], Daniel Grove[3] & William F. Jensen[3]

Over the past decade, abnormalities have been documented in white-tailed deer (*Odocoileus virginianus*) in west-central Montana. Hypotheses proposed to explain these anomalies included contact with endocrine disrupting pesticides, such as imidacloprid. We evaluated the effects of imidacloprid experimentally at the South Dakota State University Wildlife and Fisheries Captive Facility where adult white-tailed deer females and their fawns were administered aqueous imidacloprid (an untreated control, 1,500 ng/L, 3,000 ng/L, and 15,000 ng/L). Water consumption, thyroid hormone function, behavioral responses, and skull and jawbone measurements were compared among treatments. Additionally, liver, spleen, genital, and brain imidacloprid concentrations were determined by an enzyme-linked immunosorbent assay (ELISA). Results indicated that 1) control deer consumed more water than treatment groups, 2) imidacloprid was present in the organs of our control group, indicating environmental contamination, 3) as imidacloprid increased in the spleen, fawn survival, thyroxine levels, jawbone lengths, body weight, and organ weights decreased, 4) adult female imidacloprid levels in the genitals were negatively correlated with genital organ weight and, 5) behavioral observations indicated that imidacloprid levels in spleens were negatively correlated with activity levels in adult females and fawns. Results demonstrate that imidacloprid has direct effects on white-tailed deer when administered at field–relevant doses.

Neonicotinoids are a broad-spectrum insecticide predominantly used as seed dressings on major field crops and are additionally used as sprays in crop production, in managing household pests, and in deterring pests on domesticated animals[1]. Neonicotinoids derive their toxicity from agonistically binding to nicotinic acetylcholine receptors (nAChRs) on the post-synaptic nerve membrane and firing nerve impulses in a manner that is uncontrollable and uninterrupted[1–7]. Neonicotinoids were first developed in the 1990s[8], gained popularity from 2003-2011[8], and are now the most widely used pesticides in the world[10].

Popularity of neonicotinoids is due to their advertised high toxicity to insects and low toxicity to vertebrates[1]. Additionally, neonicotinoids have gained popularity by their ability to systemically protect plants while reducing application inputs for farmers[10]. In 2014, over 3.3 million kg of neonicotinoids (including acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid, and thiamethoxam) were used in the United States (excluding Hawaii and Alaska) on pasture hay, alfalfa, orchards, grapes, rice, vegetables, fruit, cotton, wheat, soybeans, corn, and other crops[11]. In South Dakota, more than 94% of U.S. corn and at least 50% of U.S. soybeans[12] are treated with one of the three neonicotinoids: clothianidin, imidacloprid, or thiamethoxam[13–15].

Neonicotinoids are widely found in the environment for numerous reasons. First, only a small quantity (2–20%) of the seed-coated insecticide is absorbed by the developing plant; the remainder is released into the environment through leaching, drainage, run-off, or snowmelt[16,17]. Neonicotinoids are highly water soluble[18];

[1]Department of Natural Resource Management, South Dakota State University, Brookings, SD, USA. [2]Ecdysis Foundation, Estelline, SD, 57234, USA. [3]North Dakota Game and Fish Department, Bismarck, ND, USA. Correspondence and requests for materials should be addressed to J.A.J. (email: jonathan.jenks@sdstate.edu)

they are prevalent in diverse water bodies in the United States, Canada, Australia, Europe, and Asia[17]. Moreover, under the right conditions, neonicotinoids can persist in the soil, sometimes for many years[1]. Finally, untreated plants associated with cropland are often contaminated by neonicotinoids due to the systemic nature of these chemicals[18]. The widespread use of neonicotinoids provides numerous opportunities for exposure to non-target, beneficial species via the water, soil, and contaminated plant tissues.

In addition to their documented effects on beneficial insects, neonicotinoids adversely affect non-target vertebrates as well, including rats (*Rattus norvegicus*: reduced sperm production, reduced offspring weight, increased abortions, skeletal abnormalities, thyroid lesions, atrophy of retina, reduced weight gain of offspring, oxidative stress, and neurobehavioral deficits), mice (*Mus musculus*: suppressed cell-mediated immune response and prominent histopathological alterations in spleen and liver), rabbits (*Sylvilagus* sp: increased frequency of miscarriage and premature births), red-legged partridges (*Alectoris rufa*: reduced adult and chick survival, fertilization rate, and immune response), Nile tilapia (*Oreochromis niloticus*: extensive disintegration of testicular tissue and changes to gonads), Medaka (*Oryzias latipes*: juvenile stress led to ectoparasite infestation), and black-spotted pond frogs (*Rana nigromaculata*: DNA damage at very low concentrations)[20]. To our knowledge, no information is available on potential effects on large mammals, such as white-tailed deer (*Odocoileus virginianus*).

Over the past decade, morphological and developmental abnormalities have been documented in white-tailed deer in west-central Montana. Of 254 male deer of various ages, 67% showed genital developmental abnormalities such as mispositioned and undersized scrota and ectopic testes[21]; these abnormalities were documented for accident-killed and injured cervids[22]. *Hoy et al.*[21] suggested that genital anomalies could be caused by endocrine disrupting pesticides but stated that, based on the information available, no cause and effect could be justified. In addition, from 2000 to 2009, brachygnathia superior (i.e., mandibular prognathia or underbite) increased from 0% to 70% for white-tailed deer that were collected from west-central (accident killed) and throughout (hunter harvested) Montana[22]. Underbite is a characteristic of congenital hypothyroidism, which has been documented in South Dakota[23], and is nearly always associated with fetal thyroid hormone function[23], but the cause has not been empirically determined for this observation.

We hypothesized that imidacloprid would have sub-lethal and potentially lethal effects on adult female and fawn white-tailed deer. We predicted that adult females, especially in the high treatment group, would have reduced Free Triiodothyronine (FT3) and Free Thyroxine (FT4) levels, presence of imidacloprid in milk, and reduced activity associated with exposure to imidacloprid. We also predicted that fawns exposed to imidacloprid at relatively high treatment levels would have abnormal genital organs, lowered FT3 and FT4 levels, reduced activity, and a high prevalence of under bite.

Methods

Our research study was conducted at the South Dakota State University Wildlife and Fisheries Captive Research Facility in Brookings County, South Dakota (44°20′N, 96°47′W). This facility housed white-tailed deer (beginning in about 1998) on 4 ha; the facility is double fenced with 3-m high woven wire. The facility is situated adjacent to agricultural fields normally planted to corn or soybeans, and is surrounded by a shelterbelt of trees. Mean annual temperatures were 7.4 °C (ranged from −28.8 °C and 34.4 °C) and 7.8 °C (−35 °C to 32.8 °C) in 2015 and 2016, respectively. Additionally, daily annual precipitation was 0.18 cm (0.03 cm to 5.2 cm) and 0.2 cm (from 0.3 cm to 7 cm) in 2015 and 2016, respectively. Finally, daily annual snowfall was 0.3 cm (0.3–17.8 cm) and 0.3 cm (0.3–11.4 cm) in 2015 and 2016, respectively[24].

Twenty adult white-tailed deer were randomly selected for the experiment and bred; parturition occurred in May and June of each experimental year. Adult females were separated into four treatments (care was taken to separate adult females so that age and weight were uniformly distributed): control (n = 4), low (n = 4), moderate (n = 5), and high (n = 7) (the moderate and high treatment groups had a larger sample size to reduce the standard error for our response variable). Deer were housed in pens of similar size (control = 130 m^2/deer, low = 175 m^2/deer, moderate = 123 m^2/deer, high = 112 m^2/deer in 2015 and 165 m^2/deer in 2016). All deer were fed rations that included soy hulls, shelled corn, and alfalfa hay *ad libitum*.

Adult females were administered aqueous imidacloprid (Product # 37984, Sigma Aldrich St. Louis, MO) from May until October to mimic free water availability within the Dakotas. We added 0 ng/L, 1,500 ng/L, 3,000 ng/L, and 15,000 ng/L of imidacloprid to the control, low, moderate, and high treatments, respectively. The low and moderate concentrations were similar to wetland levels found in groundwater in Wisconsin (detected in 24% of the groundwater sample and ranged from 260–3,340 ng/L); however, they were greater than levels found in rural streams in Iowa (detected in 23% of streams sampled and ranged from <2–42.7 ng/L) or in Canadian (Saskatchewan) wetlands (detected in 12% of wetlands and ranged from 7.1–256 ng/L)[25]. Our high treatment was intended to invoke an effect and therefore, was much greater than documented in free water. Deer were provided with a 60.6 L tub that contained 37.8 L of water treated with the appropriate amount of imidacloprid depending on the group (control, low, moderate, high). Deer consumed the water treated with imidacloprid *ad libitum*. Water levels were checked daily and refilled with the appropriate imidacloprid treated water when empty or less than 3 cm from the bottom (every 1–2 d) of containers. When refilling occurred, each tub was rinsed thoroughly and excess water was poured into 189 L tubs provided by the SDSU Environmental Health and Safety office.

Fawns born to adult females in the study were included in our experiment. On the day of parturition, each fawn was handled minimally with gloves to determine body mass and sex; fawns also were fitted with ear tags. To mimic natural water availability, fawns were not prevented from consuming the imidacloprid in water. Facilities and techniques for research were approved by the South Dakota State University Institutional Animal Care and Use Committee (IACUC number 15-055 A) and followed guidelines by the American Society of Mammologists[26].

Solution consumed. During experiments, water tubs housing aqueous imidacloprid were weighed daily to determine the volume of water consumed per group. Analysis of variance (ANOVA) was performed to compare

water consumption among treatment and control groups with date used as a covariate. To detect imidacloprid concentrations as the imidacloprid water was consumed, a 3-d experiment was conducted. On day 1, the appropriate treatment or control group concentrations were created in five, 63 L galvanized tubs. On day 2, 50% of the water was removed from all tubs. On the third day, nearly all water was removed from the tubs, leaving only enough water to coat the bottom of tubs. Samples (15 mL) were collected daily from each tub. This procedure mimicked water level reductions due to deer consumption. Imidacloprid samples were analyzed using ELISA (enzyme-linked immunosorbent assay; Abraxis, Warminister, PA; See Section 4.7 for procedures).

Collection of blood samples. Blood samples were collected from adult females and fawns in treatments using BD Vacutainer Serum tubes (Becton, Dickinson, and Company, Franklin, NJ). We collected up to 12 mL of blood from the saphenous vein approximately monthly during treatments while deer were held in a chute (Priefert Wildlife Equipment Deer Chute; Priefert®, Mount Pleasant, TX). We collected blood samples (1–10cc from the saphenous or jugular) from fawns twice; 1 wk after parturition and at approximately 5 mo of age. Blood samples were refrigerated until processed to extract serum (1 h to 2 d). Upon reaching the lab, blood samples were centrifuged (Ultra-8V; LW Scientific, Lawrenceville, GA) for 15 min at 280 × g to separate serum for testing FT3 (free triiodothyronine) and FT4 (free thyroxine) hormones.

FT3 and FT4 thyroid hormones reflect the ability of the deer to utilize body fat reserves, regulate basal metabolic rate, and control thermal regulation[27]. Serum from blood samples was transferred to labeled 1.5 mL microcentrifuge tubes (BrandTech® Scientific Inc., Essex, CT), sealed, and frozen at −20 °C. These samples were then overnighted to the Diagnostic Center for Population and Animal Health at Michigan State University (Lansing, MI) for FT3 and FT4 testing. These assays were performed with commercially available solid-phase radioimmunoassay kits (FREE T3 Solid Phase Component System and Free T4 Solid Phase Component System, MP Biomedicals Diagnostics Division Orangeburg NY 10962). The volumes of sample, assay standards, and radioligand were used according to the manufacturer's protocol. Incubation times for free T3 and free T4 assays were 2.5 h and 1.5 h, respectively, at 37 °C.

Behavioral Observations. Focal sampling of behavioral observations were collected on treatment and control groups prior to death. Behaviors included eat, lay, lay/groom, lay/ruminate, stand/ruminate, run, stand, stand/groom, stand/nurse, and walk; for fawns, the behaviors lay/curl and lay/sleep also were recorded. Observations were conducted in 1 h blocks using an ethogram[28]. During time blocks, occurrences of behaviors were tallied and the duration of each behavior (in s) was recorded. Observations occurred between 6:00 and 16:00. In each session, an adult female or fawn was randomly chosen (without replacement) from each treatment and control group (n = 28 h for 2016 fawns and n = 21 h for adult females).

Necropsies. All deer in the experiment (adult females and fawns) were euthanized and subsequently necropsied using IACUC approved protocols. Fawns were euthanized at the end of each field season (October 2015 and 2016) and adult females were euthanized at the completion of the study (October 2016). Adult females and fawns were first tranquilized using xylazine (Bayer, Englewood, Colorado) and telezol (Zoetis, Parsippany-Troy Hills, New Jersey) when held in a Priefert deer chute and, once immobilized, were euthanized using euthanasia solution (MWI Veterinary Supply, Boise, ID) according to manufacturer's suggested dosage. Once does and fawns were euthanized, they were frozen at −20 °C. All fawns and does in the experiment were necropsied at the South Dakota Animal Disease Research and Diagnostic Laboratory, South Dakota State University, Brookings, South Dakota.

Necropsies were performed by Dr. David Knudsen (assisted by E. Hughes Berheim). Liver, brain, spleen, and genital organs were extracted, weighed, and 2.54 cm^3 samples were collected. Additionally, we collected fawn jawbones to determine length. Organ samples were then frozen at −20 °C until they could be analyzed using ELISA.

ELISA Testing. Imidacloprid levels were determined for each organ collected. Brain, liver, spleen, and genital samples were removed from the freezer, and a portion of each organ (0.5–0.75 g) was minced using a sterilized scalpel and placed into a polypropylene micro centrifuge tube. Water was added to the tube at a ratio of 1 mL:1 g tissue sample. Each mixture was shaken using a vortex (Thermo Scientific), heated in an 80 °C water bath for 10 min, and frozen at −20 °C. Frozen mixtures were thawed and centrifuged (Centrifuge 5424, Eppendorf) at 21,130 g for 1 min. The liquid was extracted and placed into separate micro centrifuge tubes; remaining solids in the organ samples and remaining liquid were refrozen. Liquid samples were vortexed and a 25 µL portion was extracted and placed into a separate microcentrifuge tube. The excess liquid also was stored frozen. The remaining liquid was mixed with 25 µL of water, vortexed for 5 s, and centrifuged for 2 s in preparation for the ELISA assay.

All samples were read at 450 nm using a microplate reader (uQuant, Biotek Instruments, Winooski, VT). Each plate had at least two standard curves of purified imidacloprid (Product number: 37894 SIGMA-ALDRICH, St. Louis, MO, USA). In preparation for the standard curve on each plate, samples from negative adult females were mixed together to account for the matrix effect of the organs and a stock solution of imidacloprid was created at 0.0, 0.03, 0.06, 0.13, 0.25, 0.5, 1.0, and 2.0 ppb. The standard curve on the ELISA plate contained 25 µL control organ in solution with 25 µL of the stock solution of imidacloprid, creating eight wells with concentrations that comprised one standard curve. We were unable to use the control organs in our standard curve because our experiment was unintentionally contaminated with imidacloprid; therefore, we used the deer sample with the lowest absorbance value as our baseline for quantifying imidacloprid quantities. We optimized the tissue preparation approach for this ELISA on solid samples using peer-reviewed methods[29–34].

Analysis. Data collected in experiments were analyzed using Systat 13 (Systat Software Inc., San Jose, CA). Male and female fawn organ concentrations for those fawns that survived versus those that died were compared using t-tests. Our ELISA results indicated that there was contamination of our control group. As a consequence,

Group	Date	Total Liters Consumed (SEM)	Average Liters Per Day (SEM)	Average Liters Per Doe (SEM)	Average Liters Per Fawn (SEM)
Low	2015	1616.7 (0.61)	12.7 (0.6)	3.3 (0.2)	1.72 (0.1)
Moderate	2015	2345.9 (0.78)	17.5 (0.78)	3.5 (0.2)	2.42 (0.1)
High	2015	2606.8 (1.13)	20.8 (1.13)	3.1 (0.2)	3.98 (0.2)
Control	2015	2574.1 (0.78)	19.5 (0.78)	5.2 (0.2)	4.23 (0.3)
Low	2016	3216.5 (9.7)	17 (0.84)	4.2 (0.2)	2.78 (0.1)
Moderate	2016	2323.2 (8.9)	17.7 (0.78)	3.6 (0.1)	3.89 (0.2)
High	2016	2430.3 (13.2)	19.1 (1.2)	3.3 (0.2)	6.06 (0.3)
Control	2016	2295.2 (8.6)	18.4 (0.77)	4.7 (0.2)	4.81 (0.2)

Table 1. Water consumed by deer in treatments in 2015 and 2016 field seasons (May to October). Average liters consumed, average liters consumed per day, and average liters consumed daily per doe were recorded for each treatment and control group. Average water consumed daily by fawns is also included in the table as it was used as a covariate in the ANOVA analysis of water consumption between treatment and control groups.

in addition to ANOVA, ordinary least square (OLS) linear regression was used to assess relationships between imidacloprid concentrations in all organ samples and the response variables birth weight, fawn age, FT3, FT4, jawbone length, and organ weights; alpha was set at 0.05.

Data collected on behavioral observations for adult females and fawns were analyzed separately but combined over observation period (morning and afternoon). Furthermore, we separated deer into three groups (high, moderate, and low) based on organ imidacloprid concentrations. Finally, we used Chi-square tests to determine significant differences among behaviors observed in high, moderate, and low imidacloprid groups for adults and fawns. If Chi-square tests were significant, we used confidence intervals (90%) to assess which behaviors differed among groups (high, moderate, and low).

Results

Doe and Fawn Survival. A total of 24 and 39 fawns was born in 2015 and 2016, respectively. In 2015, 12 of the fawns were born in August and September due to late breeding. Number of single, twin, triplet, and quadruplet litter sizes, respectively, per treatment were 1, 4, 1, 1 (control), 5, 3, 0, 0 (low), 2, 3, 2, 0 (moderate), and 2, 7, 2, 0 (high); 3 fawns were found outside treatment pens and were not included in analyses. Sex ratio (females:males) of fawns was 0.46:0.54 and did not differ by treatment ($X^2_3 = 2.98$, $p = 0.394$). In 2016, a control female died and was replaced with another adult female, totaling 21 adult females in our experiment. Fawn and adult female survival decreased over the two field seasons: survival of fawns was 75% and 62% in 2015 and 2016, respectively. Of 20 adult females in 2015, 0% died (100% survival); in 2016, 19% of 21 adult females died (n = 4, 81% survival). Survival of fawns did not differ ($p > 0.05$) between 2015 and 2016. Additionally, sample size for adult females was too small to evaluate change in survival between the two field seasons.

Imidacloprid Solution Consumption. Water consumption rates in 2015 and 2016 were monitored, and daily consumption was recorded. There were significant interactions in water consumption between treatment and date in 2015 ($F_{15,419} = 2.22$, $p = 0.01$) and 2016 ($F_{15,555} = 2.19$, $p = 0.01$). In 2015, when the control was removed from the analysis, date was still significant ($F_{3,427} = 21.48$, $p = 0.01$) relative to consumption; however, water consumption per adult female was similar across treatments ($F_{2,127} = 0.60$, $p = 0.55$), indicating the control group consumed significantly more water than the treatment groups. In 2016, when excluding the control group, the high treatment group consumed less water per adult female than the low and moderate groups ($F_{2,421} = 12.83$, $p = 0.01$), even though consumption of water increased throughout the field season ($F_{5,421} = 14.25$, $p = 0.01$) (Table 1).

Necropsy Data. Organ weights were collected from adult females and fawns, and jawbone measurements were collected solely from fawns. Adult females had mean organ weights of 159 ± 8 g for brain, 809 ± 104 g for liver, 388 ± 41 g for spleen, and 87 ± 28 g for genitals. Mean organ weights of fawns were 106 ± 3.9 g for brain, 413 ± 37 g for liver, 102 ± 11.4 g for spleen, and 6 ± 0.9 g for genitals. Female fawn mean organ weights were 96 ± 6 g for brain, 342 ± 55 g for liver, 95 ± 18 g for spleen, and 3 ± 0.6 g for genitals. Male fawn mean organ weights were 115 ± 5 g for brain, 479 ± 48 g for liver, 109 ± 14 g for spleen, and 9 ± 1 g for genitals (Table 2). Average jawbone length results were 13.8 ± 0.4 cm.

ELISA Results. ELISA results indicated imidacloprid was found in the control group organs (Table 3), indicating that our treatments were contaminated; there were no significant differences across treatments for liver ($F_{3,18} = 0.511$, $p = 0.14$), brain ($F_{3,18} = 0.058$, $p = 0.388$), genital ($F_{3,18} = 0.17$, $p = 0.286$), or spleen ($F_{3,18} = 0.17$, $p = 0.328$) in adult females, or liver ($F_{3,43} = 0.04$, $p = 0.943$), brain ($F_{3,43} = 0.018$, $p = 0.576$), genital ($F_{3,43} = 0.05$, $p = 0.707$), or spleen ($F_{3,43} = 0.20$, $p = 0.199$) in fawns; gender of fawns was not significant ($p > 0.07$) in these analyses. However, imidacloprid concentration in spleen samples of adults approached significance ($r = 0.36$, $p = 0.06$) when regressed versus control and treatment categories. Nevertheless, this changed our focus from separating ELISA results by treatments to viewing the results relative to concentration of imidacloprid. Mean imidacloprid values in organs for all adult females were 0.42 ± 0.07 ng/g for liver, 0.06 ± 0.05 ng/g for brain, 0.11 ± 0.04 ng/g for spleen, and 0.69 ± 0.05 ng/g for genital (Table 3). Mean imidacloprid values in organs for female fawns were

	Brain (g) (SEM)	Liver (g) (SEM)	Spleen (g) (SEM)	Genital (g) (SEM)
Adult female	161 (8)	1015 (63)	408 (40)	64 (15)
Fawn	105 (3.9)	432 (35)	103 (11)	5 (0.8)
Male fawn	115 (5)	479 (47)	109 (14)	9 (1)
Female fawn	95 (6)	385 (53)	99 (17)	3 (0.9)

Table 2. Mean organ (brain, liver, spleen, and genital) weights (g) of adult females and fawns including standard error. Sample sizes are as follows: adult female n = 21, fawn n = 61 for the brain, spleen, and genital and n = 62 for the liver, male fawns n = 30 for brain, spleen, genital, and n = 31 for the liver, and female fawns n = 31 (all organs).

Age/Sex	Group	Survived/died	Liver (ng/g) (SEM)	Brain (ng/g) (SEM)	Spleen (ng/g) (SEM)	Genital (ng/g) (SEM)
AF	Control	All	0.351 (0.09)	0.222 (0.22)	0.012 (0.01)	0.388 (0.12)
AF	Low	All	0.133 (0.04)	0	0.077 (0.05)	0.380 (0.11)
AF	Moderate	All	0.495 (0.18)	0.010 (0.01)	0.111 (0.11)	0.287 (0.08)
AF	High	All	0.590 (0.12)	0	0.188 (0.10)	0.218 (0.06)
AF	All	Died	0.153 (0.04)	0.277 (0.21)	0.030 (0.02)	0.191 (0.13)
AF	All	Survived	0.487 (0.08)	0.003 (0)	0.124 (0.05)	0.330 (0.04)
AF	All	All	0.423 (0.07)	0.053 (0.05)	0.106 (0.04)	0.694 (0.05)
FF	Control	All	0.416 (0.06)	0.058 (0.03)	0.156 (0.04)	0.273 (0.04)
FF	Low	All	0.450 (0.05)	0.053 (0.02)	0.114 (0.02)	0.402 (0.04)
FF	Moderate	All	0.357 (0.05)	0	0.126 (0.02)	0.174 (0.02)
FF	High	All	0.426 (0.12)	0.008 (0)	0.294 (0.13)	0.222 (0.04)
FF	All	Died	0.443 (0.09)	0	0.268 (0.06)	0.219 (0.03)
FF	All	Survived	0.401 (0.07)	0.044 (0.03)	0.177 (0.08)	0.290 (0.06)
FF	All	All	0.417 (0.06)	0.028 (0.02)	0.210 (0.05)	0.264 (0.04)
MF	Control	All	0.681 (0.10)	0.065 (0.02)	0.323 (0.03)	0.162 (0.03)
MF	Low	All	0.350 (0.04)	0	0.057 (0.01)	0.168 (0.05)
MF	Moderate	All	0.566 (0.08)	0.044 (0.02)	0.252 (0.07)	0.148 (0.04)
MF	High	All	0.532 (0.09)	0.057 (0.04)	0.176 (0.06)	0.157 (0.03)
MF	All	Died	0.654 (0.08)	0.086 (0)	0.489 (0.07)	0.259 (0.04)
MF	All	Survived	0.518 (0.08)	0.057 (0.03)	0.116 (0.03)	0.113 (0.03)
MF	All	All	0.553 (0.07)	0.046 (0.02)	0.193 (0.04)	0.146 (0.03)
Fawn	All	Died	0.528 (0.04)	0.062 (0)	0.342 (0.03)	0.232 (0.02)
Fawn	All	Survived	0.463 (0.05)	0.051 (0.02)	0.144 (0.04)	0.200 (0.03)

Table 3. Average imidacloprid levels in organs (ng of imidacloprid per gram of tissue) liver, brain, spleen, and genital in adult females (AF, n = 21), fawns (n = 65), female fawns (FF, n = 32), and male fawns (MF, n = 32) per treatment and control groups. AF, FF, and MF are also separated into averages for those that were dead, and alive at the end of the experiment, and the sum of all AF, FF, or MF in our study.

0.42 ± 0.06 ng/g for liver, 0.03 ± 0.02 ng/g for brain, 0.21 ± 0.05 ng/g for spleen, and 0.26 ± 0.04 ng/g for genital (Table 3). Mean imidacloprid values in organs for male fawns were 0.55 ± 0.07 ng/g for liver, 0.05 ± 0.02 ng/g for brain, 0.19 ± 0.04 ng/g for spleen, and 0.15 ± 0.03 ng/g for genital (Table 3).

Analyses. Spleen concentrations of imidacloprid were significantly higher ($T_{59} = 2.76$, $p = 0.007$) in fawns that died compared to the fawns that survived. However, an outlier of 1.49 ng/g of spleen tissue was removed from analyses (mean of data with outlier 0.20, range 0–1.49; mean of data without outlier 0.18, range 0–0.91 ng/g of tissue); the revised result also was significant ($T_{58} = 4.36$, $p < 0.001$) (Fig. 1). The fawn with this high spleen imidacloprid concentration survived, which was not consistent with the overall trend in the data. Mean imidacloprid in spleens of fawns that died was 0.33 ± 0.26 ng/g whereas imidacloprid in spleens of fawns that survived averaged 0.10 ± 0.14 ng/g. Birth weight was not correlated with imidacloprid levels in any of the organs evaluated (Table 4). Fawn body weight at death was negatively correlated with imidacloprid levels in the spleen ($F_{1,55} = 8.22$, $p = 0.005$) and genital organs ($F_{1,56} = 4.26$, $p = 0.04$) (Table 4). Fawn age at death was correlated with imidacloprid levels in the spleen ($F_{1,57} = 10.5$, $p = 0.002$) but not in any of the other organs evaluated (Table 4). Adult female FT3 and FT4 values were not correlated with imidacloprid levels in organs (Table 4). Fawn FT3 values were not correlated with imidacloprid concentrations in organs; however, FT4 values in fawns were negatively correlated ($F_{1,59} = 7.48$, $p = 0.0092$, Table 4) with spleen imidacloprid concentrations. Adult female organ weights were negatively correlated with imidacloprid concentrations in genitals ($F_{1,19} = 3.00$, $p = 0.04$) but not with other organ levels evaluated. Fawn organ weights were negatively correlated with spleen ($F_{1,57} = 8.78$, $p = 0.0044$) and genital

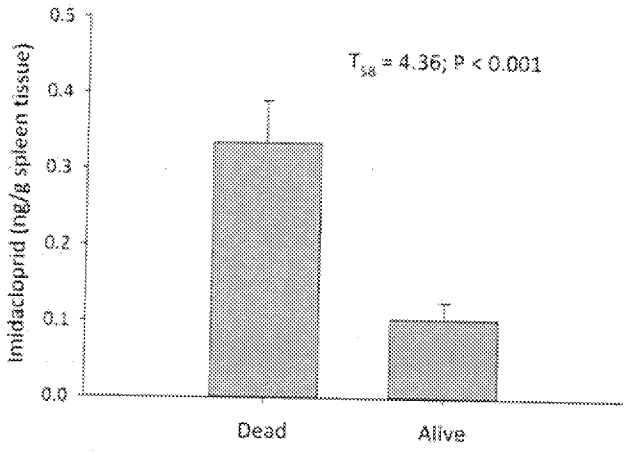


Figure 1. Average Imidacloprid levels (ng/g) in spleen tissue of 2015 and 2016 fawns (n = 62) that died prematurely compared to those that survived. Imidacloprid levels differed between those that were dead compared to alive.

Physical Responses	Brain	Liver	Spleen	Genital
Imidacloprid Concentration				
Birth Weight	$F_{1,60} = 0.04$, P = 0.83	$F_{1,60} = 0.25$, P = 0.61	$F_{1,58} = 1.25$, P = 0.26	$F_{1,53} = 0.08$, P = 0.77*
Fawn Body Weight at Death	$F_{1,57} = 0.88$, P = 0.32	$F_{1,56} = 0.35$, P = 0.55	$F_{1,55} = 8.22$, P = 0.0058*	$F_{1,56} = 4.36$, P = 0.04
Fawn Age (in Days)	$F_{1,58} = 1.78$, P = 0.18	$F_{1,60} = 0.0008$, P = 0.97	$F_{1,58} = 10.5$, P = 0.0019*	$F_{1,58} = 1.71$, P = 0.19
AF FT3	$F_{1,58} = 2.96$, P = 0.10	$F_{1,59} = 0.04$, P = 0.85	$F_{1,57} = 1.89$, P = 0.18	$F_{1,53} = 0.04$, P = 0.83
AF FT4	$F_{1,58} = 4.1$, P = 0.09	$F_{1,59} = 0.09$, P = 0.76	$F_{1,58} = 1.30$, P = 0.27	$F_{1,54} = 0.57$, P = 0.46
Fawn FT3	$F_{1,56} = 0.41$, P = 0.52	$F_{1,59} = 2.9$, P = 0.09	$F_{1,57} = 0.74$, P = 0.39	$F_{1,53} = 0.20$, P = 0.65
Fawn FT4	$F_{1,58} = 0.01$, P = 0.90	$F_{1,60} = 0.0002$, P = 0.98	$F_{1,59} = 7.48$, P = 0.009*	$F_{1,55} = 0.017$, P = 0.89
AF Organ Weights	$F_{1,58} = 0.04$, P = 0.84	$F_{1,59} = 1.15$, P = 0.3	$F_{1,57} = 0.29$, P = 0.6	$F_{1,53} = 5.0$, P = 0.04*
Fawn Organ Weights	$F_{1,58} = 2.42$, P = 0.12	$F_{1,60} = 0.10$, P = 0.75	$F_{1,57} = 8.78$, P = 0.004*	$F_{1,54} = 5.55$, P = 0.02*
Fawn Jawbone Length	$F_{1,58} = 1.5$, P = 0.22	$F_{1,60} = 0.11$, P = 0.73	$F_{1,57} = 9.98$, P = 0.002*	$F_{1,53} = 2.38$, P = 0.12

Table 4. Results of regression analyses for imidacloprid concentrations in organ samples and physical results: birth weight, fawn body weight, fawn age, FT3 and FT4, organ weights, fawn jawbone length. P-values were considered significant when < 0.05. *Indicates P-values that are significant and indicates a negative correlation so as imidacloprid increases the physical response decreases.

($F_{1,53} = 5.55$, p = 0.021) (Table 4) imidacloprid concentrations. Fawn jawbone length was negatively correlated with imidacloprid values in the spleen ($F_{1,57} = 9.98$, p = 0.002) but not with other organ concentrations (Table 4).

Imidacloprid concentrations in spleen were correlated with fawn survival; therefore, we explored relationships between spleen imidacloprid concentrations and deer behavior. Adult female (n = 21) imidacloprid concentrations in spleen were separated into low (n = 12, range = 0), moderate (n = 4, range 0.056–0.224), and high groups (n = 5, range = 0.248–0.909); the duration of behaviors were compared among groups (all spleens that had 0 ppb concentration were placed in the low group). The low imidacloprid group differed (90% CI) from the high group in the behaviors eat (groups; high = 2.4%, low = 6%), lay (high = 27%, low = 19%), lay/groom (high = 7%, low = 3%), stand/ruminate (high = 1%, low = 2%), run (high = 1%, low = 5%), and stand/groom (high = 8%, low = 5%) indicating that adult deer in the low group had higher activity levels than those in the high group. The moderate group also differed from the low group in the behaviors eat (group; moderate = 10%, low = 6%), lay (moderate = 4%, low = 19%), lay/ruminate (moderate = 2%, low = 5%), stand/ruminate (moderate = 1%, low = 2%), run (moderate = 1%, low = 5%), stand (moderate = 34%, low = 23%), stand/groom (moderate = 13%, low = 5%), and stand/nurse (moderate = 0%, low = 2%); indicating variation in behavior between the two groups (Table 5).

Fawn spleen concentrations of imidacloprid (n = 38) also were placed in low (n = 20, range = 0), moderate (n = 9, range = 0.053–0.121), and high (n = 9, range = 0.148–0.786) groups and the durations of particular behaviors were compared among groups. The low group differed (90% CI) from the high group in the behaviors lay (group; high = 43%, low = 24%), run (high = 0%, low = 4%), stand (high = 16%, low = 22%), stand/groom (high = 2%, low = 6%), and walk (high = 9%, low = 15%); indicating that the high group was less active than the low group. The moderate group also differed from the low group in the behaviors eat (group; moderate = 12%, low = 8%), lay/curl (moderate = 1%, low = 6%), and lay/groom (moderate = 15%, low = 8%); indicating variation in behavior between the two groups (Table 5).

A.F./Fawn	Group	Eat	Lay	Lay/Curl	Lay/Slp	Lay/Grm	Lay/Rum	Sta/Rum	Run	Stand	Sta/Grm	Sta/Nur	Walk
AF	High	2%*	27%*	N/A	N/A	7%*	4%	1%*	1%*	25%	8%*	1%	24%
AF	Moderate	10%*	4%*	N/A	N/A	2%	2%*	1%*	1%*	34%*	13%*	0%*	32%
AF	Low	6%	19%	N/A	N/A	3%	3%	2%	3%	23%	5%	2%	30%
Fawn	High	7%	43%^	8%	2%	7%	7%	N/A	0%^	16%^	2%^	N/A	9%^
Fawn	Moderate	12%^	20%	1%^	1%	15%^	5%	N/A	3%	21%	3%	N/A	17%
Fawn	Low	8%	24%	6%	2%	8%	5%	N/A	4%	22%	6%	N/A	15%

Table 5. Behavioral observations closest to individual adult female (AF; n = 21) and fawn (n = 38) deaths (time ranged from 1 week to 2 months) were compared to their spleen imidacloprid concentrations. Not all fawns had observations collected as: 1) fawn observations were only collected in 2016 and 2) some fawns died prior to an observation being completed. Behavioral observations are separated into three groups (low, moderate, high) according to spleen organ concentrations (with the high group having the greatest imidacloprid levels and the low group having the lowest). Behavioral observations eat, lay, lay/groom (lay/grm), lay/ruminate (lay/rum), stand/ruminate (sta/rum), run, stand, stand/groom (sta/grm), stand/nurse (sta/nur), and walk (additionally for fawns the behaviors Lay/Curl and Lay/Slp (lay/sleep)) percentages were compared between spleen organ imidacloprid concentrations. *Percentages that are significantly (90%CI) different than the low group percentages in the spleen for adult females. ^Percentages that are significantly (90%CI) different than the low group percentages in the spleen for fawns.

Discussion

Our study provides the first overview of effects of imidacloprid on white-tailed deer. We documented that deer in our experiment avoided imidacloprid-contaminated water. Moreover, we discovered that fawns that died during our experiment had greater concentrations of imidacloprid in spleens compared to those that survived. Fawns with relatively high concentrations of imidacloprid in spleen and genital organs also tended to be smaller and less healthy than those with relatively low concentrations of imidacloprid in these organs. Finally, our study provides support for reduced activity of adult and fawn white-tailed deer with relatively high concentrations of imidacloprid in spleens.

ELISA results indicated that our control experimental tissues were unintentionally contaminated with imidacloprid. Potential sources of contamination included seed-treated food and vegetation. Deer were fed soy hulls and a corn, oats, and distiller's mixture *ad libitum*. Unfortunately, it was unknown if the soybeans and grains fed to our deer were from imidacloprid-treated plants. However, corn and soybeans are commonly (≥ 94% of U.S. corn, ~ 50% of U.S. soybeans[32]) coated with one of the neonicotinoid active ingredients: clothianidin, imidacloprid, or thiamethoxam[13]. Additionally, deer in our study would often reach through the fence to browse on natural vegetation. The fields adjacent to the captive wildlife facility were a matrix of agricultural crops with a corn field about 50 m north of the facility. It is unknown what pesticides were used on the corn, but it is likely that there was a seed treatment of imidacloprid or clothianidin. In Indiana, neonicotinoid dust was documented to disperse as far as 100 m from the site[33]. Imidacloprid from fields could be washed off during rain events and be absorbed by other plants, although this transfer is poorly understood[18,34]. Therefore, uptake of imidacloprid by vegetation adjacent to the facility is a likely source of this contamination.

Water containing imidacloprid was avoided by deer in treatments in our experiment as evidenced by variable concentrations of the neonicotinoid in captive deer. Deer that avoided consumption of treated water likely drank rain water, which was available (up to 0.3 m deep) after storm events during our experiment. Research on cervid avoidance of imidacloprid is unavailable, but avoidance of imidacloprid has been recorded in red-legged partridge (*Alectoris rufa*) when offered treated seeds[35]. Other animals detect and avoid toxins in their diets; for example, kudus (*Tragelpahus imberbis*), impalas (*Aepyceros melampus*), and goats (*Capra aegagrus hircus*) in South Africa avoided plants with 5% condensed tannins during the wet season[36], likely due to the astringency of these compounds.

Significantly higher concentrations of spleen imidacloprid levels were found in fawns that died compared to those that survived. The spleen produces white blood cells that fight infection and synthesize antibodies[39]. Imidacloprid can reduce the production of spleen lymphocytes[40–42], which results in an impaired immune system[38]. Therefore, immune suppression in our fawns caused by imidacloprid likely was a factor in their deaths. Complimentary results were found in the FT4 values that are a pre-cursor to FT3 hormone, which is instrumental in regulating basal metabolic rate and thermal regulation in deer[37]. FT4 was inversely correlated with imidacloprid in spleens of fawns. Reduced metabolic rate in fawns with relatively high concentrations of imidacloprid likely explain the lower activity documented in captive deer.

Imidacloprid values in brain were low to undocumented, which was surprising considering that the pesticide affects the central nervous system; we hypothesize that this could be due to an inability of the chemical to cross the blood-brain barrier. The California Environmental Protection Agency found that imidacloprid penetrates the blood-brain barrier. However, Gupta *et al.*[43] found high imidacloprid quantities in rat liver, kidney, lung, and skin, but concentrations in the brain were low. Additionally, Krieger[44] noted that the blood-brain barrier in vertebrates blocks access of imidacloprid to the central nervous system, which reduces toxicity.

Fawns had similar birth weights regardless of the level of imidacloprid in their organs. Similarly, in Sprague-Dawley rats, there were no differences in litter size or weight gain in the offspring whether or not mothers were given an intraperitoneal injection of imidacloprid[45]. Additionally, Gawade[45] found no significant

	Liver		Spleen	
	North Dakota	Captive	North Dakota	Captive
Maximum	8.42	1.36	6.61	1.48
Minimum	0	0	0	0
Average	1.32	0.46	0.60	0.18
STD	1.68	0.34	1.12	0.26
SEM	0.10	0.03	0.06	0.02

Table 6. Comparison of liver and spleen imidacloprid concentrations (ng/g of tissue) between North Dakota free-ranging deer (n = 367) and our captive facility deer (n = 86).

difference in weights of imidacloprid exposed Wister rat pups. However, imidacloprid levels in spleen and genital tissues were negatively associated with body weight in fawns at the time of death.

FT3 and FT4 results are indicative of basal metabolic rate and thermoregulation[27]. Fawn and adult female FT3 values were similar to those reported in other studies, but FT4 results were elevated compared to previous studies[27,46–48]. We do not believe that this was the result of imidacloprid, as this pesticide decreases thyroid function in rats[49], Indian wild birds[50], and fish[51]. Rather, the elevated FT4 values may be due to a combination of pregnancy in adult females, the time of year, and artificial feed. Hamr *et al.*[52] found that thyroid hormones of artificially fed deer were elevated compared to deer that consumed natural browse. Additionally, this study also found that hormones were increased in the spring and summer. Bahnak *et al.*[53] documented that pregnant, penned deer have elevated levels of thyroid hormones.

As imidacloprid increased in the spleen, we noted that FT4 levels and spleen size decreased. As stated previously, imidacloprid has been shown to decrease FT4 levels in other vertebrates[48–51]. Additionally, research on rats has shown that organ weights (specifically liver and spleen) decrease as imidacloprid treatment increases[54–56]. From our observations and previous research, we predict that imidacloprid is suppressing the immune function and size of the spleen.

As imidacloprid increased in the genital organs of fawns and adult females, weights of the genital organs decreased. Vohra and Khera[56] found that, as oral consumption of imidacloprid increased, the ovaries of lab rats became smaller but the uterus increased in size. Additional research has shown that liver and spleen sizes will decrease as imidacloprid concentration increases; however, there was not an indication that the genital weight decreased[54–56]. Consequently, more research is needed to better understand how imidacloprid and other neonicotinoids affect reproductive tissues in mammals.

Behavioral observations indicated that high concentration of imidacloprid in the spleen resulted in less activity in adult females and fawns. This finding was similar to results on female rats and their offspring that showed significant decreases in grip time as imidacloprid concentrations from intraperitoneal injection increased, an indication of fatigue[55]. Rat movement was similarly impaired as imidacloprid (via oral consumption) increased[55,57].

Samples of liver and spleen organs were collected from white-tailed deer brought to the NDGF Wildlife Health Laboratory for a variety of reasons (e.g., illegal harvest investigations, disease, deer-vehicle collisions) from 2009–2017 throughout North Dakota.; imidacloprid concentrations were evaluated in 367 samples using the same ELISA methods as in our captive experiment. Results indicated that levels of imidacloprid in liver samples were 2.8 times higher in free-ranging deer in North Dakota [average 1.32 (0.10)] than in livers of our captive deer [average 0.46 (0.03)], Table 6. Furthermore, concentrations of imidacloprid in spleen samples from free-ranging deer in North Dakota [0.60 (0.06)] were 3.5 times higher than those in spleens of captive deer [0.17 (0.02), Table 6] in our experiment. Deer exposure to imidacloprid averaged 52.3 ± 4.6% over the years 2009 to 2017. For those free-ranging deer in North Dakota exposed to imidacloprid, average concentrations in spleens increased ($r = 0.22$, $p = 0.002$) an average of 0.11 ng/g per year from 2009 to 2017. Furthermore, 77.5% of these deer had imidacloprid levels in spleens equal to or above 0.33 ng/g (i.e., mean level of imidacloprid in spleens of fawns in captivity that died in our experiment). These results indicate that wild populations of deer exposed to imidacloprid are potentially experiencing effects similar to those seen in our captive facility experiment; i.e., reduced activity in adult females and fawns, and specifically in fawns, decreased survival, size, and health. Consequently, additional research is needed to confirm these relationships in free-ranging deer in agricultural landscapes where imidacloprid and other neonicotinoid insecticides are utilized.

Data Availability

Data are available for upload upon the publication of our manuscript.

References

1. Goulson, D. An overview of the environmental risks posed by neonicotinoid insecticides. *Journal of Applied Ecology* **50**, 977–987 (2013).
2. Tomizawa, M. & Yamamoto, I. Binding of neonicotinoids and the related compounds to the insect nicotinic acetylcholine receptor. *Journal of Pesticide Science* **17**, 231–236 (1992).
3. Buckingham, S., Lapied, B., Corronc, H. & Sattelle, F. Imidacloprid actions on insect neuronal acetylcholine receptors. *The Journal of Experimental Biology* **200**, 2685–2692 (1997).
4. Matsuda, K. *et al.* Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends in Pharmacological Sciences* **22**, 573–580 (2011).
5. Rose, P. H. Nicotine and the Neonicotinoids in *Mammalian toxicology of insecticides* (ed. Marrs, T. C.) 184–220 (Marrs, 2012).
6. Van Dijk, T. C., Staalduinen, M. A. & Van der Sluijs, J. P. Macro- invertebrate decline in surface water polluted with imidacloprid. *Plos One* **8**, e62374 (2013).

7. Sánchez-Bayo, F. P., Tennekes, H. A. & Goka, K. Impact of systemic insecticides on organisms and ecosystems in *Insecticides-Development of Safer and More Effective Technologies* 365–414 (2013).
8. Tokumato, J. M. *et al.* Effects of exposure of clothianidin on the reproductive system of male quails. *Journal of the Veterinary Medical Science* **75**, 753–760 (2013).
9. Douglas, M. R. & Tooker, J. F. Large-Scale Deployment of Seed Treatments has Driven Rapid Increase in Use of Neonicotinoid Insecticides and Preemptive Pest Management in U.S. Field Crops. *Environmental Science & Technology* **49**, 5088–5097 (2015).
10. Jeschke, P., Nauen, R., Schindler, M. & Elbert, A. Overview of the Status and Global Strategy for Neonicotinoids. *Journal of Agricultural and Food Chemistry* **59**, 2897–2908 (2011).
11. United States Geological Survey [USGS]. Estimated Annual Agricultural Pesticide Use. Pesticide Use Maps. https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2015&map=IMIDACLOPRID&hilo=L&disp=Imidacloprid (2017).
12. Stokstad, E. Pesticides under fire for risks to pollinators. *Science* **340**, 674–676 (2013).
13. Main, A. R. *et al.* Widespread use and frequent detection of neonicotinoid insecticides in wetlands of Canada's Prairie Pothole Region. *Plos One* **9**, e101400 (2014).
14. Seagraves, M. P. & Lundgren, J. G. Effects of neonicotinoid seed treatments on soybean aphid and its natural enemies. *Journal of Pest Science* **85**, 125–132 (2012).
15. Bredeson, M. M., Reese, R. N. & Lundgren, J. G. The effects of insecticide dose and herbivore density on tri-trophic effects of thiamethoxam in a system involving wheat, aphids, and ladybeetles. *Crop Protection* **69**, 70–76 (2015).
16. Mason, R., Tennekes, H., Sánchez-Bayo, F. & Jepsen, P. U. Immune Suppression by Neonicotinoid Insecticides at the Root of Global Wildlife Declines. *Journal of Environmental Immunology and Toxicology* **1**, 3–12 (2013).
17. Maloney, E., Morrissey, C. A., Headley, J. & Peru, K. Cumulative toxicity of neonicotinoid insecticide mixtures to chironomus dilutus under acute exposure scenarios. *Environmental Toxicology and Chemistry* **9999**, 1–11 (2017).
18. Morrissey, C. A. *et al.* Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: a review. *Environment International* **74**, 291–303 (2015).
19. Mogren, C. L. & Lundgren, J. G. Neonicotinoid-contaminated pollinator strips adjacent to cropland reduce honey bee nutritional status. *Scientific Reports* **6**, 29608 (2016).
20. Gibbons, D., Morrissey, C. & Mineau, P. A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. *Environmental Science and Pollution Research* **22**, 103–118 (2014).
21. Hoy, J. A., Hoy, R., Seba, D. & Kerstetter, T. H. Genital abnormalities in white-tailed deer in west-central Montana: pesticide exposure as a possible cause. *Journal of Environmental Biology* **23**, 189–197 (2002).
22. Hoy, J. A., Haas, G. T., Hoy, R. D. & Hallock, P. Observations of brachygnathia superior in wild ruminants in Western Montana, USA. *Wildlife Biology in Practice* **7**, 15–29 (2011).
23. Zimmerman, T. L., Jenks, J. A., Holler, L. D., Jacques, C. N. & Morlock, W. W. Congenital Hypotrichosis in a white-tailed deer fawn from South Dakota. *Journal of Wildlife Diseases* **40**, 145–149 (2004).
24. NOAA National Weather Service Cooperator Observer Program. NOAA homepage. http://www.nws.noaa.gov/om/coop/ (2017).
25. Giorio, C. A. *et al.* An update of the worldwide integrated assessment (WIA) on systemic insecticides. Part 1: new molecules, metabolism, fate, and transport. Environmental Science and Pollution Research 1–33. https://doi.org/10.1007/s11356-017-0394-3 (2017).
26. Sikes, R. and the Animal Care and Use Committee of the American Society of Mammologists. Guidelines of the American Society of Mammologists for the use of wild mammals in research and education. *Journal of Mammalogy* **97**, 663–688 (2016).
27. Bergman, E. J., Doherty, P. F., Bishop, C. J., Wolfe, L. L. & Banulis, B. A. Herbivore body condition response in altered environments: mule deer and habitat management. Plos One **9**:e106374, http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0106374 (2014).
28. Altmann, J. Observational study of behavior: sampling methods. *Behavior* **49**, 227–267 (1974).
29. Pecenka, J. R. & Lundgren, J. G. Non-target effects of clothianidin on monarch butterflies. *Science of Nature* **102**(3/4), 19 (2015).
30. Bredeson, M. M. & Lundgren, J. G. The effect of a thiamethoxam seed treatment on pests and yield in cultivated sunflowers. *Journal of Economic Entomology* **108**, 2665–2671 (2015).
31. Bredeson, M. M., Reese, R. N. & Lundgren, J. G. The effects of insecticide dose and herbivore density on tri-trophic effects of thiamethoxam in a system involving wheat, aphids, and lady beetles. *Crop Protection* **69**, 70–76 (2015).
32. Mogren, C. L. & Lundgren, J. G. Neonicotinoid-contaminated pollinator strips adjacent to cropland reduce honey bee health. *Scientific Reports* **6**, 29068 (2016).
33. Pecenka, J. R. & Lundgren, J. G. The importance of dung beetles and insect communities on degradation of cattle dung pats in eastern South Dakota. *PeerJ* **6**, e5220 (2018).
34. Bredeson, M. M. & Lundgren, J. G. Thiamethoxam seed treatments reduce foliar predator and pollinator populations in sunflowers (Helianthus annuus), and extra-floral nectaries as a route of exposure for seed treatments to affect the predator, Coleomegilla maculate (Coleoptera: Coccinnellidae). *Crop Protection* **106**, 85–92 (2018).
35. Krupke, C. H., Holland, J. D., Long, E. Y. & Eitzer, B. D. Planting of neonicotinoid-treated maize poses risks for honey bees and other non-target organisms over a wide area without consistent crop yield benefit. *Journal of Applied Ecology* **54**, 1449–1458 (2017).
36. Bonmatin, J. M. *et al.* Environmental fate and exposure; neonicotinoids and fipronil. *Environmental Science and Pollution Research* **22**, 35–67 (2015).
37. Lopez- Antia, A., Ortiz-Santaliestra, M. E. & Mateo, R. Experimental approaches to test pesticide treated seed avoidance by birds under a simulated diversification of food sources (2014).
38. Cooper, S. M. & Owen-Smith, N. Condensed tannins deter feeding by browsing ruminants in a South African savanna. *Oecologia* **67**, 142–146 (1985).
39. Mebius, R. E. & Kraal, G. Structure and function of the spleen. *Nature Publishing Group* **5**, 606–616 (2005).
40. Mohany, M., Badr, G., Refaat, I. & El-Feki, M. Immunological and histological effects of exposure to imidacloprid insecticide in male albino rats. *African journal of Pharmacy and Pharmacology* **5**, 2108–2114 (2011).
41. Gawade, L., Dadarkar, S. S., Husain, R. & Gatne, M. A detailed study of developmental immunotoxicity of imidacloprid in Wistar rats. *Food and Chemical Toxicology* **51**, 61–70 (2013).
42. Mohany, M., El-Feki, M., Refaat, I., Garraud, O. & Badr, G. Thymoquinone ameliorates the immunological and histological changes induced by exposure to imidacloprid insecticide. *The Journal of Toxicological Sciences* **37**, 1–11 (2012).
43. Gupta, R. C. & Milatovic, D. Insecticides. Pages 389–407 *in* R. C. Gupta, editor. Biomarkers in Toxicology. Elsevier Inc. San Diego, California, USA.
44. Krieger, R. 2010. Hayes' Handbook of Pesticide Toxicology. Third Edition. Academic Press Cambridge Massachusetts, USA (2014).
45. Abou-Donia, M. B. *et al.* Imidacloprid induces neurobehavioral deficits and increases expression of glial fibrillary acidic protein in the motor cortex and hippocampus in offspring rats following in utero exposure. *Journal of Toxicology and Environmental Health* **71**, 119–30 (2008).
46. Watkins, B. E., Nachreiner, R. F., Schmitt, S. M., Cooley, T. M. & Ullrey, D. E. Thyroid function in fed and fasted white-tailed deer fawns. *Canadian Journal of Zoology* **60**, 1331–1338 (1981).
47. Creekmore, T. E. & Creekmore, L. H. Health assessment of white-tailed deer of the Cuyahoga Valley National Recreation Area, U.S. Department of the Interior. Ohio Madison, WI, U.S (1999).

48. Bishop, C. J., Watkins, B. E., Wolfe, L. L. & White, G. C. Evaluating mule deer body condition using serum thyroid hormone concentrations. *The Journal of Wildlife Management* **73**, 462–467 (2009).
49. Abbassy, M. A., Marzouk, M. A., Nasr, H. M. & Mansy, A. S. Effect of imidacloprid and tetraconazole on various hematological and biochemical parameters in male albino rats. *Journal of Political Sciences & Public Affairs* **2**, 122. https://doi.org/10.4172/2332976.1000122 (2014).
50. Pandey, S. P. & Mohanty, B. The neonicotinoid pesticide imidacloprid and the dithiocarbamate fungicide mancozeb disrupt the pituitary–thyroid axis of a wildlife bird. *Chemosphere* **122**, 227–234 (2014).
51. Priya, P., Maruthi, Y. A. & Chaitanya, D. A. Proceedings of the second International Summit on Integrative Biology: Effect of imidacloprid insecticide stress on levels of serum thyroid hormones and minerals of fresh water fish clarias batrachus (walking cat fish): interaction with cortisol. Hilton-Chicago/Northbrook, Chicago, USA (2014).
52. Hamr, J. & Bubenik, G. Seasonal thyroid hormone levels of free-ranging white-tailed deer in Ontario. *Canadian Journal of Zoology* **68**, 2174–2180 (2011).
53. Balmak, B. R., Holland, J. C., Verme, L. J. & Ozoga, J. J. Seasonal and nutritional influences on growth hormone and thyroid activity in white-tailed deer. *The Journal of Wildlife Management* **45**, 140–147 (1981).
54. Vohra, P., Khera, K. S. & Sangha, G. K. Physiological, biochemical and histological alterations induced by administration of imidacloprid in female albino rats. *Pesticide Biochemistry and Physiology* **110**, 50–56 (2014).
55. Memon, S. A., Memon, N., Mal, B., Shaikh, S. A. & Shah, M. A. Histopathological changes in the gonads of male rabbits on exposure to imidacloprid insecticide. *Journal of Entomology and Zoology Studies* **2**, 159–163 (2014).
56. Vohra, P. & Khera, K. S. Effect of imidacloprid on Reproduction of Female Albino Rats in Three Generation Study. *Journal of Veterinary Science & Technology* **7**, 340, https://doi.org/10.4172/2157-7579.1000340 (2016).
57. Najafi, G., Razi, M. & Feyzi, S. The effect of chronic exposure with imidacloprid insecticide on fertility in mature male rats. *International Journal of Fertility and Sterility* **4**, 9–16 (2010).

Acknowledgements

Funding for this study was provided by the South Dakota Agricultural Experiment Station and Federal Aid to Wildlife Restoration (Study No. 3M5842) administered through South Dakota Department of Game, Fish and Parks. Funding for analysis of free-ranging deer in North Dakota was provided by North Dakota Department of Game and Fish. Special thanks to technicians C. Lee, B. Becher, J. Christenson, A. Wieseler, S. Carstens, and J. Jensen at South Dakota State University and R. Herigstad, M. Kietzman, J. Mortenson, H. Prichert, and A. Pachl at North Dakota Game and Fish Department.

Author Contributions

Berheim, collected data, wrote paper; Jenks obtained funding, conducted analyses, edited paper; Lundgren, conducted laboratory analyses; Michel, provided support for data collection and analysis; Grove, obtained funding and provided samples for analysis; Jensen, obtained funding and provided samples for analysis.

Additional Information

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

November 13, 2017

Comments from the Natural Resources Defense Council
On the Imidacloprid: Human Health Draft Risk Assessment
for Registration Review (June 2017)
Document ID EPA-HQ-OPP-2008-0844-1235[1]

Comments submitted electronically to Docket ID: EPA-HQ-OPP-2008-0844-1234

The Natural Resources Defense Council (NRDC) has no direct or indirect financial or fiduciary interest in the manufacture or sale of any chemical or methodology that would be the subject of these comments.

These comments are on the Imidacloprid Human Health Draft Risk Assessment (2017), herein referred to as EPA HHDRA.

BACKGROUND

Imidacloprid is one of the most popular and widespread insecticides in the U.S. The neonicotinoid or "neonic" pesticides are used to treat soil, seeds and foliage to control sucking insects such as rice hoppers, aphids, thrips, whiteflies, turf insects, soil insects and some beetles.[2] Imidacloprid is most commonly used on the following crops: rice, cereal, corn, potatoes, vegetables, sugar beets, fruit, cotton, hops and turf.[3] Apart from its use for crops, it is also used in and around homes, yards, gardens, and public parks and green spaces to control termites, and fleas on pets.[4]

[1] https://www.regulations.gov/document?D=EPA-HQ-OPP-2008-0844-1235
[2] Imidacloprid. Extoxnet: Extension of Toxicology Network.
http://pmep.cce.cornell.edu/profiles/extoxnet/haloxyfop-methylparathion/imidacloprid-ext.html
[3] Imidacloprid. Extoxnet: Extension of Toxicology Network.
http://pmep.cce.cornell.edu/profiles/extoxnet/haloxyfop-methylparathion/imidacloprid-ext.html.
[4] Imidacloprid: General Fact Sheet. 2010. National Pesticide Information Center.
http://npic.orst.edu/factsheets/imidagen.html#whatis

Imidacloprid is also used as a seed treatment – a use that NRDC opposed in our comments to EPA on its preliminary aquatic risk assessment for imidacloprid (July 2017).[5] The value to farmers of these costly and environmentally-damaging seed treatments is increasingly shown to be negligible or marginal at best. A 2015 multi-state University Extension report sharply questioned both the economic and environmental justifications for using neonicotinoid-treated seed in soybeans: "To summarize: For typical field situations, independent research demonstrates that neonicotinoid seed treatments do not provide a consistent return on investment.[6] [7] [8] The current use of neonicotinoid seed treatments in soybean and other crops far exceeds pest pressures."[9] Since then, reports by academics[10], the Center for Food Safety (2017)[11], and EPA experts (2014)[12] have provided further evidence to support these conclusions. Worse, the toxic soil residues from neonic seeds may make pest control even more difficult by disrupting biological control systems. [13, 14] Indeed, a study by Douglas et al (2015) reported that in slug-infested fields, soybean grown with neonic-treated seeds had poorer yields than counterparts grown without neonic seeds.[15]

---

[5] see NRDC comments on EPA Preliminary Aquatic Risk Assessment, July 2017. EPA-HQ-OPP-2008-0844-1146. Available at https://www.regulations.gov/document?D=EPA-HQ-OPP-2008-0844-1219

[6] Hodgson, E.W., and G. VanNostrand. 2014. 2014 Yellow Book Report of insecticide evaluation for soybean pests, 21 pp. Department of Entomology, Iowa State University, Publication 296-14. Hodgson, E.W., and G. VanNostrand. 2013. 2013 Yellow Book Report of insecticide evaluation for soybean pests, 25 pp. Department of Entomology, Iowa State University, Publication 294-13. Hodgson, E.W., and G. VanNostrand. 2012. 2012 Yellow Book Report of insecticide evaluation for soybean pests, 35 pp. Department of Entomology, Iowa State University, Publication 291-12. As reported in: http://ento.psu.edu/extension/field-crops/fact-sheet-Effectiveness-of-Neonicotinoid-Seed-Treatments-in-Soybean

[7] Seagraves, M.P., and J.G. Lundgren. 2012. Effects of neonicotinoid seed treatments on soybean aphid and its natural enemies. Journal of Pest Science. 85: 125-132.

[8] McCarville, M.T., M.E. O'Neal, B.D. Potter, K.J. Tilmon, B.P. McCornack, J.F. Tooker, and D.A. Prischmann-Voldseth. 2014. One gene versus two: A regional study on the efficacy of single gene versus pyramided resistance for soybean aphid management. Journal of Economic Entomology. 107: 1680-1687

[9] Bailey, Wayne et. al. 2015. The Effectiveness of Neonicotinoid Seed Treatments in Soybean." Purdue Extension Service (and others), E-268. December. http://ento.psu.edu/extension/field-crops/fact-sheet-Effectiveness-of-Neonicotinoid-Seed-Treatments-in-Soybean

[10] Krupke CH, Holland JD, Long EY, and Eitzer BD. (2017). Planting of neonicotinoid-treated maize poses risks for honey bees and other non-target organisms over a wide area without consistent crop yield benefit. J Appl Ecol.

[11] Center for Food Safety report 2017. Alternatives to Neonicotinoid Insecticide-Coated Corn Seed: Agroecological Methods are better for Farmers and the Environment. http://www.centerforfoodsafety.org/issues/304/pollinators-and-pesticides/press-releases/4957/landmark-report-shows-bee-killing-seed-coatings-arent-worth-the-harm

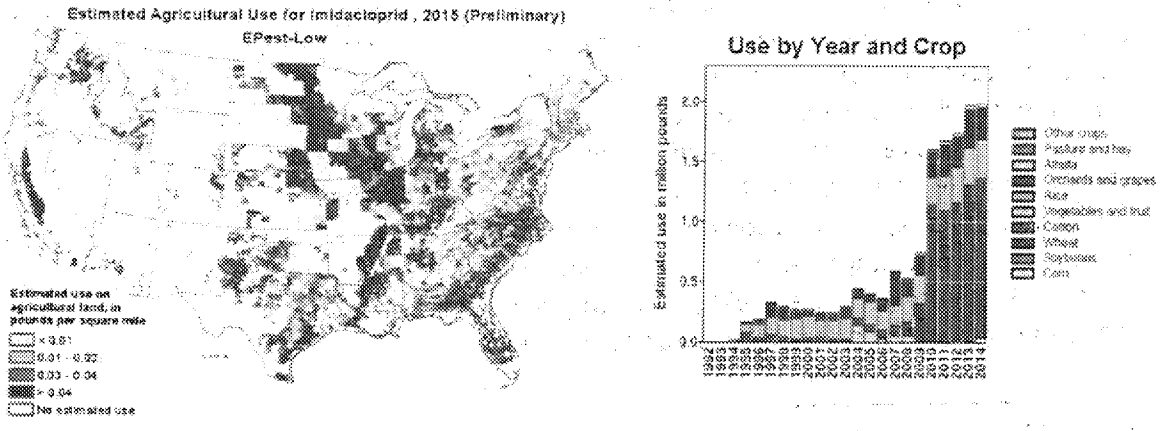
[12] EPA 2014. Benefits of Neonicotinoid Seed Treatments to Soybean Production. https://www.epa.gov/sites/production/files/2014-10/documents/benefits_of_neonicotinoid_seed_treatments_to_soybean_production_2.pdf

[13] Seagraves, M.P., and J.G. Lundgren. 2012. Effects of neonicotinoid seed treatments on soybean aphid and its natural enemies. Journal of Pest Science. 85: 125-132.

[14] Douglas, M.R., J.R. Rohr, and J.F. Tooker. 2014. Neonicotinoid insecticide travels through a soil food chain, disrupting biological control of non-target pests and decreasing soybean yield. Journal of Applied Ecology.

[15] Douglas, M.R. and J.F. Tooker. 2015. Large scale deployment of seed treatments has driven rapid increase in use of neonicotinoid insecticides and preemptive pest management in US field crops. Environmental Science and Technology.

Figure: U.S. Geological Survey map of estimated agriculture use of imidacloprid for 2015[16]
Note: State-based and other restrictions on pesticide use may be reflected in the EPest-low estimates because they are based primarily on surveyed data.



Imidacloprid kills insects by debilitating their central nervous system. It mimics nicotine and binds to nicotinic acetylcholine receptors, blocking the receptor and thereby preventing nerve cell transmission, leading to paralysis and death in insects. In humans these receptors are found in neuromuscular junctions and the central nervous system.[17] Imidacloprid is a systemic insecticide. When plants take it up through soil or leaves, it spreads to other parts of the plant, such as its stems, fruits and flowers. Insects that chew or suck on these plants ingest the insecticide, which attacks their nervous system and kills them.[18] Neonic pesticides also are absorbed into the plant tissue of milkweed – the sole food source for monarch butterfly larvae - rendering the milkweed deadly to the caterpillars and other beneficial pollinators. [19]

Imidacloprid residue on baby and children's foods

New and disturbing evidence shows that imidacloprid is making its way into our food and water supply. The United States Department of Agriculture (USDA) Pesticide Data Program (PDP) monitors imidacloprid residues in food. According to the Pesticide Action Network publicly searchable database, "What's On My Food", using data aggregated from public sources

[16] USGS. National Water-Quality Assessment (NAWQA) Project. Estimated Annual Agricultural Pesticide Use. Pesticide Use Maps - Imidacloprid.
https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2015&map=IMIDACLOPRID&hilo=L&disp=Imidacloprid

[17] Imidacloprid: General Fact Sheet. 2010. National Pesticide Information Center.
http://npic.orst.edu/factsheets/imidagen.html#whatis.

[18] Imidacloprid: General Fact Sheet. 2010. National Pesticide Information Center.
http://npic.orst.edu/factsheets/imidagen.html#whatis

[19] Pecenka, J.R., and J.G. Lundgren. 2015. Non-target effects of clothianidin on monarch butterflies. Science of Nature. 102: 19.

including the USDA PDP 2012 data, imidacloprid was detected in the following baby foods and common children's foods: baby food - applesauce (0.3% of samples); baby food - pears (13.6% of samples); bananas (1.8% of samples); apples (20% of samples); cherries (14% of samples); and grapes (48% of samples).[20] Detection rates were greater in fresh, unprocessed fruits and vegetables. Moreover, the pesticide's systemic nature means it cannot just be washed off the surface of these foods prior to consumption.

Imidacloprid in water

A study by the U.S. Geological Survey found widespread neonic contamination of streams across the country. At least one neonic pesticide was detected in half the samples collected, with imidacloprid being the most frequently detected contaminant (37% of samples).[21] Interestingly, clothianidin and thiamethoxam concentrations were higher in agriculture areas, whereas imidacloprid concentrations were higher in denser urban areas within the watershed. This suggests that its urban uses in lawns and gardens, and parks and public spaces are having a measurable impact on watershed contamination, including sources of tap water. Imidacloprid was reported in tap water in Iowa City, causing alarm for residents.[22] EPA has not established a mandatory drinking water limit (Maximum Contaminant Level, MCL) for imidacloprid or any of the other neonic pesticides.

Imidacloprid exposure from residential uses

Imidacloprid is approved for a wide range of residential uses that lead to exposures to vulnerable populations, including women of reproductive age, infants, and toddlers. It is permitted to be used on ornamental turf and plants, residential lawns and gardens, pets (spot-on treatments and collars), bed bugs, crack-and-crevice treatments, and on wood as a preservative and termiticide.

Advantage® is a popular anti-flea treatment applied topically to cats and dogs. It contains 9.1% imidacloprid. In one study, significant amount of transferable residue was found on the dog's coat up to 4 weeks after a single treatment.[23] According to the authors, Advantage® may pose health risks for individuals who regularly handle pets such as veterinarians, pet owners and caretakers.[24]

---

[20] PAN. What's On My Food. Publicly searchable database, Pesticide Action Network. http://www.whatsonmyfood.org/index.jsp

[21] Hladik, M.L., and Kolpin, D.W., 2015, First national-scale reconnaissance of neonicotinoid insecticides in streams across the USA: Environmental Chemistry, doi:10.1071/EN15061. Online here: https://toxics.usgs.gov/highlights/2015-08-18-national_neonics.html

[22] Jaxen J. 2017. Breaking Study: Insecticides Found in Iowa Drinking Water. GreenMed Info. http://www.greenmedinfo.com/blog/breaking-study-insecticides-found-iowa-drinking-water.

[23] Craig MS, Gupta RC, Cander TD, and Britton DA. 2005. Human exposure to imidacloprid from dogs treated with Advantage®. Toxicol Mech Methods. 15(4):287-91.

[24] Craig MS, Gupta RC, Cander TD, and Britton DA. 2005. Human exposure to imidacloprid from dogs treated with Advantage®. Toxicol Mech Methods. 15(4):287-91.

Imidacloprid poisoning incident data gathered by the EPA between 1992 and mid-2009 documents over 22 thousand minor poisoning incidents in people from residential uses.[25] Most people were exposed from applying imidacloprid products to lawns and gardens, or by playing with pets that had recently been treated for fleas and ticks with imidacloprid-containing products. Reported adverse effects included skin irritation and rashes, numbing and tingling on fingers and lips, facial numbness and swelling, lethargy and nausea. Over the same time EPA documented over 4 hundred deaths of domestic animals, mainly dogs and cats treated with flea and tick products.

A study by NIH-funded researchers from UNC Chapel Hill and UC Davis reported that frequent exposure (self-reported by parents) to imidacloprid applied as flea and tick treatments for pets (Advantage by Bayer) during pregnancy was associated with an up to 4-fold elevated risk of Autism Spectrum Disorder (OR 2.0, 95% CI 1.0-3.9) in prenatally-exposed children.[26]

These studies together support the use of an FQPA factor of at least 10X to prevent harm from prenatal and early life exposures to imidacloprid.

FQPA – EPA FAILED TO ADDRESS RISKS TO CHILDREN

Under the Federal Food, Drug and Cosmetics Act ("FFDCA"), EPA may not "establish or leave in effect a tolerance for a pesticide chemical residue in or on a food" unless the Administrator determines that the tolerance is safe. 21 U.S.C. § 346a(b)(2)(A)(i). The Food Quality Protection Act ("FQPA"), a 1996 amendment to the FFDCA, requires that EPA make an affirmative determination that there is reasonable certainty of no harm from use of a pesticide in accordance with its label, and it must make this finding considering aggregate and cumulative exposures to infants and children. Id. § 346a(b)(2)(C)(ii)(I), (II). EPA must revoke a tolerance if it finds a pesticide residue would not be safe. Id. § 346a(b)(2)(A)(i).

EPA HHDRA (2017) determined that the Food Quality Protection Act (FQPA) factor could be removed (reduced to 1X), based on the following reasons:

1. EPA finds that the toxicological database is adequate, there are no major data gaps;
2. EPA believes that the observed neurotoxic effects are well characterized and protected for;
3. EPA believes that all fetal and offspring effects are well characterized and protected for; and

[25] US EPA. Specified Incidents for Registered Products Containing a Specified Active Ingredient Imidacloprid, Submitted January 1, 1992 through June 26, 2009. FOIA Request #HQ-RIN-01475-09 to Jennifer Sass, NRDC. June 19, 2009.

[26] Keil AP, Daniels JL, Hertz-Picciotto I. Autism spectrum disorder, flea and tick medication, and adjustments for exposure misclassification: the CHARGE (CHildhood Autism Risks from Genetics and Environment) case-control study. Environ Health. 2014 Jan 23;13(1):3.

4. EPA believes that the exposure assessments are sufficient and unlikely to underestimate exposure.

We challenge each of the above four assertions as follows:

1. EPA's assessment has significant data gaps by failing to address the non-industry studies in the published scientific literature;
2. EPA failed to address evidence of developmental risks, including neurobehavioral and endocrine risks;
3. EPA does not characterize or protect for fetal and offspring effects;
4. All pet collar uses pose an unacceptable risk to children; they should be cancelled immediately. Further, EPA's methods are likely a significant underestimate of risk.

Accordingly, EPA should apply an FQPA factor of at least 10x to imidacloprid. Our detailed response is below.

1. EPA's assessment has significant data gaps by failing to address the non-industry studies in the published scientific literature

EPA fails to cite or address studies from the peer-reviewed published literature, which support the need for an FQPA factor of at least 10X. Here we describe some of those studies.

Laboratory tests with cell cultures and rodents led the European Food Safety Authority (EFSA) to categorize two neonics – imidacloprid and acetamiprid – as possibly impairing the developing human nervous system.[27] Non-industry published animal studies report neurobehavioral impairments in rodents that were exposed to imidacloprid prenatally, from a single high-dose injection of the pesticide to the pregnant rat (337 mg/kg at day 9 of pregnancy).[28]

There are some published medical reports that can be used by EPA to understand the clinical sequelae of acute imidacloprid poisoning in people:

- A healthy 35-year-old male farmer ingested 350 mL of imidacloprid in an attempted suicide.[29] After thirty minutes, he began to feel drowsiness, severe nausea and copious vomiting. After he was admitted to hospital, he showed bradycardia and had a cardiorespiratory arrest. He did not respond to advance life support, and died.

[27] EFSA Assesses Potential Link Between Two Neonicotinoids and Developmental Neurotoxicity. December 17, 2013. http://www.efsa.europa.eu/en/press/news/131217

[28] Abou-Donia MB, Goldstein LB, Bullman S, Tu T, Khan WA, Dechkovskaia AM, Abdel-Rahman AA. Imidacloprid induces neurobehavioral deficits and increases expression of glial fibrillary acidic protein in the motor cortex and hippocampus in offspring rats following in utero exposure. J Toxicol Environ Health A. 2008;71(2):119-30.

[29] Shadnia S and Hassanian-Moghadd H. 2008. Fatal intoxication with imidacloprid insecticide. American Journal of Emergency Medicine. 26. 634.e1 – 634.e4.

- In another case, a 69-year-old woman ingested 200 mL Confidor, which contained 9.6% imidacloprid. She also became drowsy and started to vomit within 30 minutes. After two hours, she suffered a fatal heart fibrillation, and died 12 hours later.[30]
- A 24-year-old farmer accidentally inhaled an insecticide spray containing 17.8% imidacloprid while working on his farm. He became disoriented and then unconscious, which lead to further exposure. Subsequently, he suffered extreme agitation, frothy secretions, cyanosis, diaphoresis, and disorientation. The authors concluded "Clearly, there is an urgent need for higher toxicity rating and accumulation of human data for this compound, especially for subclinical neuropsychiatric effects."[31]
- A study of Sri Lankan self-poisoning incidents reported on 68 patients (61 were poisoned from self-ingestion, and 7 from dermal exposure). Symptoms presented in approximately four hours after exposure, and included symptoms of nausea, vomiting, headache, dizziness, abdominal pain, and diarrhea.[32]
- A study of Taiwan National Poison Control Center reports identified 53 cases of acute poisoning. Among them, two died from respiratory failure.[33] The authors note that the clinical signs and symptoms presented much like cholinergic poisoning.

A published study of rural residents reported an association between residential proximity to agricultural use of imidacloprid and anencephaly.[34] While the study is limited by the potential for exposure misclassification and by the small number of cases that were exposed, it is consistent with effects reported for other related pesticides, and highlights the need for precautionary measures to prevent prenatal exposures, and for more study.

Earlier this year, a team of government and academic researchers published a systematic review of studies on human health effects of the neonic pesticides, including case reports of intentional poisoning studies, studies of occupational exposures, and population epidemiologic studies.[35] Their review identified four general population studies that reported associations between chronic neonic exposure and adverse developmental or neurological outcomes, including tetralogy of Fallot (AOR 2.4, 95% CI: 1.1, 5.4), anencephaly (AOR 2.9, 95% CI: 1.0, 8.2), autism spectrum disorder [AOR 1.3, 95% credible interval (Cri): 0.78, 2.2], and a symptom

---

[30] Huang N. 2006. Fatal ventricular fibrillation in a patient with acute imidacloprid poisoning. American Journal of Emergency Medicine. 24(7):883-885.

[31] Ritesh, A. and Rajagopala, S. 2007. Severe neuropsychiatric manifestations and rhabdomyolysis in a patient with imidacloprid poisoning. American Journal of Emergency Medicine. 25(7): 884-845.

[32] Mohamed F, Gawarammana I, Robertson TA, Roberts MS, Palangasinghe C, Zawahir S, et al. 2009. Acute human self-poisoning with Imidacloprid compound: a neonicotinoid insecticide. PLoS One 4(4):e5127, doi: 10.1371/journal.pone.0005127.

[33] Phua DH, Lin CC, Wu M, Deng JF, Yang CC. 2009. Neonicotinoid insecticides: an emerging cause of acute pesticide poisoning. Clin Toxicol (Phila) 47(4):336–341.

[34] Yang W, Carmichael SL, Roberts EM, et al. Residential Agricultural Pesticide Exposures and Risk of Neural Tube Defects and Orofacial Clefts Among Offspring in the San Joaquin Valley of California. *American Journal of Epidemiology*. 2014;179(6):740-748.

[35] Cimino AM, Boyles AL, Perry MJ, et al. 2017. Effects of Neonicotinoid Pesticide Exposure on Human Health: A Systematic Review. Environmental Health Perspectives. 125(2): 155-162. http://dx.doi.org/10.1289/EHP515.

cluster including memory loss and finger tremor (AOR 14, 95% CI: 3.5, 57).[36, 37] After systematically reviewing and assessing the quality and outcome of the available studies, the authors concluded that the studies were limited but suggestive of human health impacts, and that more study was needed giving the widespread use and human exposure to this class of pesticides.[38]

These published studies can inform EPA's human health risk assessment, including its use of uncertainty factors. These data provide evidence that current residential uses of imidacloprid products lead to acute poisonings with a range of severity. Further, these data provide some evidence that chronic exposures, particularly during critical windows of development prenatally and in early childhood, may lead to persistent neurobehavioral impairments. These data are consistent with the neurotoxic evidence from animal studies, and lend support for an FQPA factor of at least 10X to prevent harmful exposures to women of reproductive age and young children.

2. EPA failed to fully characterize developmental risks, including neurobehavioral and endocrine risks

Evidence of Harm in Developmental Neurotoxicity Test (Rat)

To address concerns of early-life sensitivity to imidacloprid, the registrant, Bayer CropScience, submitted Developmental Neurotoxicity Test results (DNT) (MRID 45537501; Sheets, 2001). EPA determined at the time that Bayer's DNT study did not fulfill the guideline requirements due to serious flaws in the study design including incomplete data reporting and inadequate positive controls.[39] The original study review by EPA asked that registrants submit the following information to address the "study deficiencies": complete analytical data; morphometric measurements for caudate/putamen [brain regions] for females at intermediate doses; and, additional positive control data. EPA classified it as acceptable/non-guideline, and assigned the same NOAEL and LOAEL (20 and 55-58 mg/kg-day respectively) to both the pregnant dams and the offspring.

However, in an undated memo, EPA subsequently re-classified the Bayer DNT study as "acceptable/ guideline" without any additional data or information.[40] Given the significant lack of raw data and the inability of EPA to do a thorough and independent analysis of Bayer's study

[36] Yang W, Carmichael SL, Roberts EM, et al. Residential Agricultural Pesticide Exposures and Risk of Neural Tube Defects and Orofacial Clefts Among Offspring in the San Joaquin Valley of California. *American Journal of Epidemiology*. 2014;179(6):740-748.

[37] Keil AP, Daniels JL, Hertz-Picciotto I. Autism spectrum disorder, flea and tick medication, and adjustments for exposure misclassification: the CHARGE (CHildhood Autism Risks from Genetics and Environment) case-control study. Environ Health. 2014 Jan 23;13(1):3.

[38] Cimino AM, Boyles AL, Perry MJ, et al. 2017. Effects of Neonicotinoid Pesticide Exposure on Human Health: A Systematic Review. Environmental Health Perspectives. 125(2): 155-162. http://dx.doi.org/10.1289/EHP515.

[39] See NRDC's comments in 2009 to EPA in the federal docket (www.regulations.gov) on this issue. Document ID: EPA-HQ-OPP-2008-0772-0005 and EPA's DER of the rodent DNT, EPA-HQ-OPP-2008-0844-0116.

[40] see EPA supplemental Data Evaluation Record, memo from Anwar Dunbar and Connor Williams.

– which is inexcusable, given EPA's extensive authority under FIFRA to require data and information – the study should not be considered guideline.

Nonetheless, EPA's subsequent review increased the maternal NOAEL from 20 to 55 mg/kg/day, and noted that a LOAEL was not observed. This change meant that the offspring were determined to be 2.75X more sensitive than the adult dams. This alone should have triggered an FQPA factor of at least 3X.

In addition, EPA noted in the HHDRA that, "in the rat DNT study, there is evidence of an 'apparent' increased quantitative susceptibility" (EPA HHDRA, Section 4.4.3, page 16). This should have justified at least the remainder of the FQPA – another 3X – which would have resulted in a full 10X FQPA. Instead, in the HHDRA, the EPA dismissed the evidence by positing that it considers the concern to be low because the effects (body weight deficits and decreased motor activity) are well characterized and the regulatory limits are expected to be protective of the rat pup effects seen at higher doses.

How can EPA call the effects "well characterized" given that the registrant failed to supply many study details to EPA? The whole study was poorly characterized, as EPA initially characterized it. Further, the rat DNT study is specifically designed to identify developmental neurotoxic effects – that is the title of the test protocol. The regulatory limits that EPA set are based on a subchronic and chronic toxicity study in dogs that is not designed to identify subtle neurobehavioral or neurodevelopmental effects, and doesn't test prenatal exposures. The dog study protocol doses adult dogs only, and therefore cannot identify possible adverse impacts from prenatal or early-life exposures. The dog study is in appropriate to characterize developmental effects, and EPA has no basis for using its results to dismiss evidence of developmental harm in prenatally exposed rodents.

Developmental Toxicity Tests (Rats and Rabbits)

In addition to the DNT study, EPA (1993) reviewed two developmental toxicity studies, one in rats (83-3a, MRID 422563-38, Becker et al 1992) and one in rabbits (83-3b, MRID 422563-39). From the rat study, EPA (1993) derived a maternal NOEL of <10 mg/kg/day and LOEL of 10 mg/kg/day based on decreased body gain. At 100 mg/kg/day, decreased food consumption was observed. EPA derived a developmental NOEL of 30 mg/kg/day and LOEL of 100 mg/kg/day based on decreased body weight and increased skeletal abnormalities (wavy ribs). From the rabbit study, EPA (1993) derived a NOEL of 24 mg/kg/day and LOEL of 72 mg/kg/day for both the maternal and offspring groups. However, the maternal NOEL was based on decreased body weight, increased resorption, increased abortion, and death. The offspring NOEL was based on decreased body weight and increased skeletal abnormalities. Thus the impacts on development are much more severe than on the adult. It is worth noting that the offspring NOEL values from these two tests is almost identical to the offspring NOAEL derived from the rat DNT study (discussed above) – about 20 mg/kg-day.

Like the DNT study results, these tests also show compared with adults, the impacts of prenatal and early-life exposures to imidacloprid causes permanent and very severe impacts on exposed offspring – spontaneous abortion, death, and severe skeletal abnormalities. This very dramatic obvious difference in qualitative susceptibility between the offspring and maternal effects also supports the need to retain the default FQPA factor. In other words, prenatal and juvenile exposure to imidacloprid can have serious and possibly deadly effects compared with mild or no effects on adults.

However, given the limitations of the studies – the only one that was specifically designed to detect developmental neurotoxicity had serious limitations and lack of detailed reporting – the study is likely to bias to the null, or have false negatives (fail to identify harm) at the lower doses due to lack of sensitivity. This is because an underpowered study that fails to find an effect at the lower dose is difficult to interpret – the study may not be sensitive enough or have enough statistical power to detect a low-dose effect. However, if the same underpowered study finds an effect – the impacts in offspring at the LOAEL - it is more likely that the effect is real. As an analogy, if you reach into a haystack only a few times (an underpowered study or a low dose) and don't find a needle (a null result), you cannot conclude whether or not there may be needles in the haystack, whereas if you do find a needle (an underpowered study that finds an effect), then there is at least one needle, and probably more, in the haystack i.e. the effect is real. Thus, there is very low confidence that the reported NOAEL is adequately protective, further supporting the use of the default FQPA factor.

Developmental Neurotoxicity effects (Dog study)

EPA HHDRA (2017) emphasized that the dog is the most sensitive species that was tested, and particularly highlighted the neurotoxic effects that were reported in the sub-chronic study, including trembling and tremors at the mid and high doses (EPA HHDRA, page 14). Cal DPR (2006) noted that "unlike the mild effects seen in the chronic study, sub-chronic treatment for 4 or 13 weeks with similar doses (24-64 mg/kg/day) imidacloprid produced a marked toxicity in dogs, including mortality, severe tremors, morphological changes in liver and thyroid and weight loss" (Cal DPR p. 39-40). EPA (2017) suggests that the reason that these effects were not reported in the chronic study were differences in measurement times, which were much more frequent in the sub-chronic study. EPA notes that neurotoxicity was also reported in the rate acute and DNT studies, and in the acute neurotoxicity study, indicating strong concordance across species (EPA HHDRA, p. 14).

The dog study should be considered as supportive of the findings of developmental toxicity in offspring from the rodent and rabbit studies. However, as noted above, the dog NOAEL is not relevant to FQPA considerations, since it is an adult only study, and therefore cannot identify possible adverse impacts from prenatal or early-life exposures.

EPA Ignores Affirmative Biologically Relevant Evidence of Adverse Endocrine Effects

NRDC raised many of these same concerns of EPA disregarding evidence of endocrine effects, in our comments to EPA on its preliminary aquatic risk assessment for imidacloprid (July 2017).[41]

In a rat teratology study (83-3a, MRID 422563-38, Becker et al 1992), imidacloprid (94.2% a.i.) was administered daily by gavage to mated female Wistar rats from gestation days (GD) 6 through 15 (Becker et al., 1992). Each dose group consisted of 25 rats. The respective doses were 0, 10, 30 and 100 mg/kg/day. On gestational day 21 (GD 21) the fetuses were delivered by a cesarean section and examined for developmental abnormalities. Interestingly, CA DPR noted that "exposure of dams to 100 mg/kg/day imidacloprid resulted in a disproportionally high number of male fetuses (59%). This effect was statistically significant ($p \leq 0.05$) relative to control animals, which had approximately 1:1 ratio between males and females. Furthermore, the sex ratio in the 100 mg/kg/day group was outside the historical range (range 45-51.9% male fetuses out of 2194 fetuses; mean 49.5±1.5%). The historical database was compiled by the authors from 8 developmental toxicity studies to represent the same period of study (1986-1988)." (CA DPR p. 46). CA DPR concluded that, "Presently, it is unclear why the dams at the high-dose group had more male fetuses, since there was no post-implantation loss, e.g. selective loss of female fetuses, to account for the higher number of the male fetuses. It was speculated that imidacloprid may possess androgenic properties, causing virilization of female fetuses, which could explain the profound phenotypic gender change" (CA DPR p. 46). This study provides some evidence that imidacloprid is an endocrine disrupting chemical.

In the imidacloprid Endocrine Disruptor Screening Program (EDSP), EPA identified many adverse developmentally-related endocrine effects:

- Effects on avian reproduction (i.e., decreases in eggshell thickness and/or egg production/ hatchability) were observed in the Part 158 wildlife studies;
- Imidacloprid caused treatment-related effects on fertilization and embryonic development, mainly at zygote formation and first cleavage of the zygote (Gu et al. (2013);
- In the male pubertal assay, a 15% decrease in the weight of the dorsolateral prostate was observed at the low dose, and at the high dose, treatment related effects included decreases in accessory sex organs/tissues, pituitary weight and a delay (3.4 days) in preputial separation;
- In the amphibian metamorphosis assay (AMA), while delays in developmental stage and decreases in growth were observed.

Despite these many developmentally-related endocrine effects, EPA concluded that, "Based on weight of evidence considerations, mammalian or wildlife EDSP Tier 2 testing is not recommended for imidacloprid since there was no convincing evidence of potential interaction with the estrogen, androgen or thyroid pathways".[42] However, this ignores the affirmative

[41] see NRDC comments on EPA Preliminary Aquatic Risk Assessment, July 2017. EPA-HQ-OPP-2008-0844-1146. Available at https://www.regulations.gov/document?D=EPA-HQ-OPP-2008-0844-1219

[42] EDSP Weight of Evidence Conclusions on the Tier 1 Screening Assays for the List 1 Chemicals. June 29, 2015. Available online at https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-tier-1-screening-determinations-and

evidence of biologically relevant endocrine effects. It also ignores that these endpoints were incorporated into EDSP-1 because they were fit for the purpose of identifying specific endocrine hazards. Moreover, the EDSP-1 was never intended to characterize that hazard (dose-response) or characterize risk. Finally, it also ignores the inherent limitations in the testing methods such as failure to provide complete biological coverage of all relevant pathways, so that the tests have the potential to miss chemicals that could adversely impact large sensitive life stages (e.g., developing fetuses, infants, and children). As such, the endpoints listed above are identifying the hazard that EPA had intended in the design of the assays. Simply put, EPA should not dismiss the hazards identified by these data.

Developmental effects, including effects on endocrine systems, support the use of the default FQPA factor.

3. EPA does not characterize or protect for developmental neurotoxicity:

Consistent and supportive of FQPA requirements to provide special consideration and protection to early life stage vulnerabilities, the National Academies of Science issued its seminal report, Science and Decisions (2009) with the observation that "small chemical exposures in the presence of existing disease processes and other endogenous and exogenous exposures can have linear dose response relationships at low doses." Science and Decisions, p. 158. In other words, there may be no safe threshold in the human population for many chemicals. Newer science shows many examples of chemicals that increase the risk of various non-cancer health effects—such as reproductive harm and neurological effects—at low doses, without any scientifically-identifiable threshold (Grandjean and Landrigan 2006; Grandjean et al 2008). Even if a threshold is established in an individual, when risk is assessed across a diverse population, there is a diminishing likelihood that the same threshold exists because some people are more vulnerable than others.

The Science and Decisions report recommended that agencies use the same approach for addressing risks from both cancer and non-cancer health effects (such as developmental or reproductive effects). The committee also concluded that, "scientific and risk management considerations both support unification of cancer and non-cancer dose response assessment approaches." (Science and Decisions, p. 9). The agency called for a "unified-dose response framework" that includes a systematic evaluation of factors such as background exposures, disease processes, and inherent vulnerabilities. This evaluation will inform the choice of the appropriate dose-response model. The Science and Decisions report also pointed out that the population differs due to age, disease status, nutrition, and other factors. Because of these differences, and the fact that people are exposed to multiple chemicals, science supports using a model that does not have an assumption of a threshold (a study NOAEL or regulatory Reference Dose) below which exposures cause zero risk in the population. The Science and Decisions report recommended that a conceptual model be developed that is, "from linear conceptual models unless data are sufficient to reject low-dose linearity; and nonlinear conceptual models otherwise" (Science and Decisions, p. 144).

The National Academies report recommended that regulators assume that all exposures, even low level, are associated with some level of risk, unless there is sufficient data to the contrary, after accounting for background chemical exposures, biological make-up, and population variability.

Because of the severe and lasting harm from lead, mercury, organophosphate pesticides, and other developmental neurotoxic agents that are widely used in commerce, a coalition of expert researchers and medical health associations came together to from Project TENDR: Targeting Environmental Neuro-Development Risks.[43] The coalition issued a consensus statement supported by the following professional associations: The American College of Obstetricians and Gynecologists (ACOG); Child Neurology Society; Endocrine Society; International Neurotoxicology Association; International Society for Children's Health and the Environment; International Society for Environmental Epidemiology; National Council of Asian Pacific Islander Physicians; National Hispanic Medical Association; National Medical Association. Specific to imidacloprid, these experts also warned that, "When the federal government banned some uses of OP pesticides, manufacturers responded by expanding the use of neonicotinoid and pyrethroid pesticides. Evidence is emerging that these widely used classes of pesticides pose a threat to the developing brain (Kara et al 2015) ".[44] The TENDR consensus statement recommended that, "Evidence of neurodevelopmental toxicity of any type—epidemiological or toxicological or mechanistic—by itself should constitute a signal sufficient to trigger prioritization and some level of action."[45]

It is a reasonable scientific presumption that imidacloprid – which has endocrine disruption activity and is a developmental neurotoxicant – should warrant the use of the full 10X FQPA factor where reproductive aged women and children may be exposed.

4. All pet collar uses pose an unacceptable risk to children; they should be cancelled immediately. Further, EPA's methods are likely a significant underestimate of risk

EPA acknowledges that exposure to children 1-2 years old, the most vulnerable age group, exceed EPA's Level of Concern from the pet collar uses alone (see HHDRA Table 6.2.2., page 31).

---

[43] http://projecttendr.com

[44] Kara M, Yumrutas O, Demir CF, Ozdemir HH, Bozgeyik I, Coskun S, et al. 2015. Insecticide imidacloprid influences cognitive functions and alters learning performance and related gene expression in a rat model. Int J Exp Pathol 96(5):332–337

[45] Bennett D, Bellinger DC, Birnbaum LS, Bradman A, Chen A, Cory-Slechta DA, Engel SM, Fallin MD, Halladay A, Hauser R, Hertz-Picciotto I, Kwiatkowski CF, Lanphear BP, Marquez E, Marty M, McPartland J, Newschaffer CJ, Payne-Sturges D, Patisaul HB, Perera FP, Ritz B, Sass J, Schantz SL, Webster TF, Whyatt RM, Woodruff TJ, Zoeller RT, Anderko L, Campbell C, Conry JA, DeNicola N, Gould RM, Hirtz D, Huffling K, Landrigan PJ, Lavin A, Miller M, Mitchell MA, Rubin L, Schettler T, Tran HL, Acosta A, Brody C, Miller E, Miller P, Swanson M, Witherspoon NO; American College of Obstetricians and Gynecologists (ACOG); Child Neurology Society; Endocrine Society; International Neurotoxicology Association; International Society for Children's Health and the Environment; International Society for Environmental Epidemiology; National Council of Asian Pacific Islander Physicians; National Hispanic Medical Association; National Medical Association. Project TENDR: Targeting Environmental Neuro-Developmental Risks The TENDR Consensus Statement. Environ Health Perspect. 2016 Jul 1;124(7):A118-22.

This is the case for all pet collar scenarios, both cats and dogs of all sizes, for both the presumption of a pet collar exposure of 50%liquid/50%dust ratio, and 1%liquid/99%dust ratio. Some exposures are excessive from dermal routes of exposure alone (small cats), and all scenarios are excessive from oral routes of exposure. In summary, all pet collar uses "fail on their own" (HHDRA, p. 32), even before additional residential exposures are aggregated (including dietary and non-dietary home exposures). Given these excessive risks to children, EPA should cancel all pet collar uses of imidacloprid immediately – safer affordable and effective alternatives already exist.

EPA calculates exposures, and then compares them to the total target Margin of Exposure, MOE. Risk estimates exceed the Level of Concern (LOC) when they are below the target MOE, which EPA determined to be 100 (the product of a 10X interspecies and 10X intraspecies factor). In the HHDRA, the total MOE's for pet collar scenarios for children 1-2 years old range from 18 to 96, where the lower values carry the highest risk, and all values below the target MOE of 100 exceed EPA's LOC. For example, the MOE of 18 (collars on small cats, assuming 1%liquid/99%dust ratio) are 5.5-fold lower, and therefore exceed the LOC. Had EPA used an additional 10X FQPA – as the law requires and these comments argue for – then the target MOE would have been 1000. In this case, the MOE of 18 would have been excessive by 55-fold.

Not only has EPA determined that all pet collar uses pose excessive and unacceptably high risks to children, but EPA's methods are likely to underestimate risk in several important ways, detailed below:

EPA underestimates time with pet

Relying in its existing methods, EPA fails to protect children who spend more than 1 hour per day in contact with their pet from harmful exposures to imidacloprid products. For its standard protocols, EPA has utilized data from a single study, with only nine children, as the basis for its Exposure Time (ET) variable derivation such that the risk assessment assumes that toddlers spend only one hour per day playing with their pets. Because EPA chose to use the median of this extremely limited study, half of the children spent more than one hour per day playing with their pets and are therefore not protected by EPA's standard. Importantly, at least two of the nine children in the study spent more than an hour and a half with their pets each day, and at least one spent over two hours. EPA does not provide evidence from studies of children's interactions with their pets to support the claim that the assumptions in the 2102 Residential SOP – that one hour of exposure to the treated pet during which a child touches the pet 4 times and engages is hand-to-mouth activity- reflects a reasonable high-end estimate of time spent in contact with a pet treated with imidacloprid pet products.[46] Published studies have reported that found that, on average, children put objects in their mouth while playing 15 times per hour

[46] EPA 2015. Tetrachlorvinphos (TCVP): Responses to Arguments Presented in the Natural Resources Defense Council Inc's (NRDC) August 5, 2015 Opening Brief in *NRDC v EPA*, Case No. 15-7005 (9th Cir.) p.9

(and, at the high end, 66 times per hour).[47] In summary, EPA's repeated insistence that its SOP captures "vigorous" contact with a pet and that a child would not be expected to have this type of contact with a pet for more than 1 hour per day is not supported by any evidence. To account for the limited nature of the dataset, EPA should use the upper portion of the distribution (95-99th percentile) and assume that children spend at least two hours with their pets per day. Indeed, in other risk assessments, EPA has assumed that children spend two hours per day playing with their pets.[48]

EPA disregards additional indirect routes of exposure

EPA failed to evaluate indirect exposure routes, such as contact with a surface contaminated by pesticide residue or mouthing of objects which contain pesticide residue. There is evidence in the published literature that pet pesticides can contaminate surfaces that children can come into contact with and that children's object-to-mouth activity can result in increased exposures to chemicals.[49] Other surfaces which could reasonably be expected to have imidacloprid residues include clothing, furniture, rugs, pet bedding, and any other place in the home that comes into contact with the fur of the pet and that a child might touch with their hand or put in their mouth. In a study conducted to evaluate exposures to pesticide-impregnated flea collars, pesticide levels were measured in t-shirts worn by children who had treated pets in their house.[50] The researchers noted that two t-shirts worn were found to have significantly higher levels of pesticide residue. The authors concluded that these higher levels were the result of increases in the amount of time those children spent in direct contact with their pet dogs on those days. This finding is additional evidence that EPA's SOPs are likely an underestimate of actual exposures and therefore risks to children.

Because it is reasonable to assume that a child could encounter imidacloprid residues by both direct *and* indirect methods in a single day, the total dose experienced by the child is the sum of all the exposure pathways. EPA provides no documentation that a child's cumulative exposure to surfaces contaminated with residues resulting from contact with a treated dog or cat (i.e. the bed where the dog sleeps and/or the chair where the grooms themselves) would be so low as to not contribute to the cumulative daily exposure.

EPA's Risk Estimates for Liquid Formulations Underestimate Exposure

---

[47] Ko S, Schaefer PD, Vicario CM, Binns HJ; Safer Yards Project. Relationships of video assessments of touching and mouthing behaviors during outdoor play in urban residential yards to parental perceptions of child behaviors and blood lead levels. J Expo Sci Environ Epidemiol. 2007 Jan;17(1):47-57.

[48] *See* EPA Reregistration Eligibility Decision for Dichlorvos (DDVP) 167 (July 31, 2006).

[49] Davis, M. Keith, et al. "Assessing intermittent pesticide exposure from flea control collars containing the organophosphorus insecticide tetrachlorvinphos." *Journal of Exposure Science and Environmental Epidemiology* 1-7 (2008): 6

[50] Davis, M. Keith, et al. "Assessing intermittent pesticide exposure from flea control collars containing the organophosphorus insecticide tetrachlorvinphos." *Journal of Exposure Science and Environmental Epidemiology* 1-7 (2008): 6

We are pleased that EPA has updated its methodology for assessing pet collar uses, to consider either liquid or solid (dust) formulations, and assuming various liquid/dust ratios (HHRA p. 5, Section 6.2). This is important because EPA's assumption in its 2012 Residential Standard Operating Procedures (SOPs) that flea collars "are designed to release the active ingredient in liquid form" is not substantiated by the evidence and should not be relied upon to evaluate risk. To the contrary, in the evaluation of tetrachlorvinphos (TCVP) impregnated collars, all the documents in the docket for the registration review describe the collar formulation supports the characterization as a solid formulation. EPA summarizes this evidence in the TCVP document containing responses to the arguments raised in the NRDC's opening brief. In this document, EPA confirmed that more than half of the total pet collar products include text on the label describing the release of a powder from the collar. EPA then concludes that "some collars may act by extrusion of the active ingredient from the collar matrix as fine dust."[51] In contrast, EPA's only justification for the characterization of the collars as liquid formulations is a reference to the 2012 SOP and the explanation that this position was based upon "research conducted at the time of the SOP development." No citation or evidence is if any of the collars, currently available on the market, function in any manner that resembles a liquid.

Therefore, EPA's updated approach to evaluate the risk to children from these products must be based on the realistic assumption that exposures are predominantly from pesticide dust.

RISKS ARE EXCESSIVE USING DEFAULT 10X FQPA

As described above, an FQPA factor of at least 10X is warranted, and if this were done, acute dietary (food and drinking water) exposures (reported as the acute and chronic Population Adjusted Dose, aPAD and cPAD) would exceed the Level of Concern (LOC) for all populations, including the most vulnerable—infants, children 1-2 years old, and women of reproductive age (WORA). Chronic dietary exposures would exceed the LOC for children 1-2 years old.

(See Table below, adapted from EPA HHDRA, Table 5.4.4., page 23. All values that exceed the LOC are in bold).

	% of aPAD, using 1X FQPA	% of aPAD if EPA applies 10X FQPA	% of cPAD, using 1X FQPA	% of cPAD if EPA applies 10X FQPA
General US population	38	**380**	5.6	56
All infants (under 1 yr)	84	**840**	9.8	98
Children 1-2 yrs	93	**930**	12	**120**
Children 3-5 yrs	73	**730**	8.8	88
Children 6-12 yrs	45	**450**	5.6	56
Youth 13-19 yrs	27	**270**	4.0	40

[51] EPA 2015. Tetrachlorvinphos (TCVP): Responses to Arguments Presented in the Natural Resources Defense Council Inc's (NRDC) August 5, 2015 Opening Brief in *NRDC v EPA*, Case No. 15-7005 (9th Cir.) p.8

Adults 20-49 yrs	29	**290**	5.2	52
Adults 50-99 yrs	29	**290**	5.4	54
WORA: females 13-49 yrs	29	**290**	5.1	51

There are many well-recognized factors that can lead to a significant increased exposure to pesticides in young children compared with adults, making children more vulnerable to the harmful effects of pesticides:

- Young children spend more time crawling on the floor where pesticide dusts can settle, picking up residues on their hands and clothing, and then thumb-sucking or putting their hands in their mouths during eating, sleeping, and playing. This leads to transfer of pesticide residues from the home to the child (Hyland and Laribi, 2017; NRC 1993)
- Young children have less varied diets compared to adults, and frequently have more foods with higher levels of pesticide residues, such as fruits, fruit juices (Cohen Hubal et al., 2000; Freeman et al., 2005, 2001; Lu et al., 2006).
- Children also eat, breathe, and drink more on a per kilogram basis than adults; estimates have shown that caloric consumption is about two and a half times greater for infants than adults, when normalized for body weight (NRC, 1993).
- In addition to higher exposure levels and higher absorption rates, children also have less ability to metabolize and eliminate many chemicals (Roberts and Karr, 2012). For example, extensive research has shown that children below the age of seven have significantly lower levels of paraoxonase 1 (PON1), an enzyme that detoxifies some pesticides in humans; lowered expression of PON1, from infant exposure to neonicotinoids, has been linked to the development of Autism in North American children (D'Amelio et al., 2005).

For the above reasons, Congress directs EPA under the FQPA to add a 10X safety factor to adjust for added risks from exposures to pregnant women and young children. However, in its imidacloprid assessment, EPA removed it in full, selecting a 1X for all acute and chronic risk assessments. EPA should restore the 10x factor for all populations, especially for children and women of reproductive age.

Even without use of the legally-mandated default 10X FQPA, all pet collar uses pose an unacceptable risk to children. For this reason, EPA proposed to exclude them from the aggregate assessment (HHDRA, p. 33-34). EPA should cancel all pet collar uses effective immediately, because EPA has determined that their risks to children are excessive.

EPA VIOLATES CANCER GUIDELINES – FAILS TO ADDRESS CANCER RISKS

Properly conducted chemical carcinogenesis bioassays in animals have long been recognized and accepted as valid predictors of potential cancer hazards to people.[52] The relevance of experimental bioassays to humans rests on four well-accepted observations:

- Experimental animals and humans are mammals sharing many basic genetic, pharmacologic, toxicologic, and carcinogenic responses;
- Findings from independently conducted bioassays on the same chemicals are consistent;
- All known human carcinogens that have been tested adequately are also carcinogenic in animals and, almost without exception, share identical target sites; and
- Nearly one-third of human carcinogens were first discovered to induce cancer in animals (e.g., 1,3-butadiene, diethylstilbestrol, dioxins, ethylene oxide, 2-naphthylamine, formaldehyde, vinyl chloride), although most of these were not regulated by EPA until human evidence mounted."

Despite animal toxicology evidence to the contrary, EPA HHDRA (2017) confirmed its previous classification of imidacloprid as a "Group E" carcinogen (evidence of non-carcinogenicity in humans") by a HED RfD/Peer Review Committee (EPA 1993; EPA HHDRA 2017, p. 18). However, this predates the EPA Cancer Guidelines (2005), and should be re-done in a transparent and publicly-accessible manner.

For the reasons detailed in this section, according to EPA's own Cancer Guidelines imidacloprid should be classified as posing a cancer risk to humans based on the evidence from liver tumors in the male rats, including statistically significant adenoma/carcinomas and statistically significant rare cholangiocellular carcinomas, which is a very aggressive and often deadly cancer in humans.

According to the EPA Cancer Guidelines, characterizing a chemical as not carcinogenic is a high bar, and requires robust data, consideration of all routes of exposure, a full range of doses, and all mechanisms of action relevant to humans. For Imidacloprid, EPA did not have any evidence at all for non-carcinogenicity, and in fact wrongly dismissed affirmative evidence of carcinogenicity from robust guideline studies.

---

[52] Haseman J, Melnick R, Tomatis L, Huff J. Carcinogenesis bioassays: study duration and biological relevance. Food Chem Toxicol. 2001;39:739–744.
Huff J. Value, validity, and historical development of carcinogenesis studies for predicting and confirming carcinogenic risks to humans. In: Kitchin KT, editor. Carcinogenicity Testing, Predicting, and Interpreting Chemical Effects. New York: Marcel Dekker; 1999. pp. 21–123.
Huff J, Jacobson MF, Davis DL. The Limits of Two-Year Bioassay Exposure Regimens for Identifying Chemical Carcinogens. Environmental Health Perspectives. 2008;116(11):1439-1442.
Maltoni C. The contribution of experimental (animal) studies to the control of industrial carcinogenesis. Appl Occup Environ Hyg. 1995;10:749–760.
Tomatis L. Identification of carcinogenic agents and primary prevention of cancer. Ann NY Acad Sci. 2006;1076:1–14

The following subsections detail areas where EPA ignored elevated cancer risks, deviating from its own cancer guidelines.

Chronic/Oncogenic Oral Studies – Rat

EPA (1993) had two chronic feeding/oncogenicity studies in rats (83-1, 83-2; MRID 422563-31, 422563-32). Imidacloprid (94.3% active ingredient) was administered to Wistar rats (50/sex/group) in the diet at concentrations of 0, 100, 300, and 900 ppm (0, 5.7, 17, or 51 mg/kg/day in males and 0, 7.6, 25, or 73 mg/kg/day in females) (Eiben and Kaliner, 1991; Cal EPA 2006).[53] Interim examinations were conducted on an additional 10 rats/sex/dose after 1 year after treatment. A supplemental study to determine the maximum tolerated dose (MTD) was conducted on 50 rats/sex/dose that were either used as controls or administered imidacloprid at a concentration of 1,800 ppm (103 mg/kg/day in males and 144 mg/kg/day in females) for two years. The two studies were reviewed together by EPA, and data from the control animals were pooled together from both studies. Results are summarized in the Table below (copied from Cal DPR 2006).[54]

Tumor Incidences from 2-year Dietary Studies with Imidacloprid in Wistar Rats

	Incidence of Neoplastic Lesions									
	24 months									
	Males					Females				
Dose (ppm)	0	100	300	900	1800	0	100	300	900	1800
(mg/kg/day)	0	5.7	17	51	103	0	7.6	25	73	144
Thyroid Gland										
Number of thyroids	100	50	50	50	50	100	50	50	50	50
Parafollicular cell adenoma	5	5	6	3	0	2	4	1	0	4
Parafollicular carcinoma	0	0	1	0	0	1	0	0	1	0
Follicular adenoma	4	0	0	0	3	0	0	0	0	0
Liver										
Number of livers	100	50	50	50	50	100	50	50	50	50
Adenoma	0	0	0	0	1	2	0	0	0	0
Carcinoma	1	1	1	0	1	0	0	0	0	0
Cholangioma	0	0	0	0	0	1	0	0	0	2
Cholangiocellular carcinoma	0	0	0	0	2	0	0	0	0	0

The cholangiocellular tumors – being very rare, and being outside the historical control range – should have been considered evidence of carcinogenicity. EPA Cancer Guidelines stipulate that for rare tumors a lower standard of statistical significance should be applied (EPA 2005 Section 2.2.2.1.3).[55] The NIEHS National Toxicology Program also considers rare tumors even if their incidences do not reach significance, particularly if they are outside the historical range as was the case in this imidacloprid study. The Organisation for Economic Cooperation and

[53] Cal DPR 2006. Eiben and Kaliner, 1991 and reported in California Environmental Protection Agency Department of Pesticide Regulation (Cal DPR). 2006. Risk Characterization Document for Imidacloprid. Available at: http://www.cdpr.ca.gov/docs/risk/rcd/imidacloprid.pdf

[54] Cal DPR 2006. Table 8 in Risk Characterization Document for Imidacloprid. Available at: http://www.cdpr.ca.gov/docs/risk/rcd/imidacloprid.pdf

[55] https://www.epa.gov/sites/production/files/2013-09/documents/cancer_guidelines_final_3-25-05.pdf

Development (OECD) rules are the same, "a non-significant increase for a rare tumour may be considered a true negative if it falls within the historical control range"[56] which was not the case here, and therefore the tumors should have been seen as evidence of cancer. Moreover, from a clinical and human risk standpoint, cholangiocellular carcinoma is a very aggressive type of cancer (intrahepatic and extrahepatic liver tumor) that has no efficient screening markers so is often diagnosed in patients when it is too late to deliver life-saving treatment (the five-year survival rate is only 17-18%).[57] [58] Approximately 6 thousand new cases of this deadly cancer are diagnosed annually in the U.S. (approximately 2-3% of new cancer cases each year). This should have been seen as evidence of cancer.

The hepatic adenomas and carcinomas should have been considered as evidence of cancer risk. Liver tumors were reported in the male rats; these included 1/100 carcinoma in the control animals, 1/50 carcinoma at the lowest dose, 1/50 carcinoma in the mid-low dose, none in the mid-high dose, and 2/50 in the high dose group. This means that each of the treatment groups (except the mid-high dose) has a 2-fold higher incidence of carcinomas as the control group, and 4-fold higher in the high dose group if adenomas and carcinomas are combined. The standard practice is to combine liver adenomas and carcinomas[59] because in people adenomas can proceed to malignant carcinomas; medical standard practice is to treat them similarly, by surgical removal. Hepatocellular carcinoma is currently the sixth most common type of cancer globally, with a high mortality rate and an increasing incidence worldwide.[60]

The liver adenomas and carcinomas should not have been dismissed with the excuse that the incidence was within the historical range for the tumors. Using historical controls to dismiss tumors is a violation of the EPA Cancer Guidelines. Historical controls are generally the historical collection of tumor responses from untreated control groups from studies in the same laboratory within two to three years of the study being evaluated – the study provides no details about whether the historical control data is appropriate (from the same lab within a few years). The EPA Cancer Guidelines are clear that, "the standard for determining statistical significance of tumor incidence comes from a comparison of tumors in dosed animals with those in concurrent control animals" (EPA 2005 Section 2.2.2.1.3).[61] The Guidelines specifically state that, "statistically significant increases in tumors should not be discounted simply because incidence rates in the treated groups are within the range of historical controls or because incidence rates in the concurrent controls are somewhat lower than average." (EPA 2005 Section 2.2.2.1.3).[62] The internationally accepted OECD Guidelines also warn against using

---

[56] OECD 2010. Draft Guidance Document 116 – Section 4. Page 27. https://www.oecd.org/chemicalsafety/testing/45335229.pdf

[57] Vogel A, Saborowski A. Cholangiocellular Carcinoma. Digestion. 2017;95(3):181-185. https://www.ncbi.nlm.nih.gov/pubmed/28288474

[58] https://seer.cancer.gov/statfacts/html/livibd.html

[59] McConnell EE, Solleveld HA, Swenberg JA, Boorman GA. Guidelines for combining neoplasms for evaluation of rodent carcinogenesis studies. J Natl Cancer Inst. 1986 Feb;76(2):283-9.

[60] Schlageter M, Terracciano LM, D'Angelo S, Sorrentino P. Histopathology of hepatocellular carcinoma. World Journal of Gastroenterology : WJG. 2014;20(43):15955-15964.

[61] https://www.epa.gov/sites/production/files/2013-09/documents/cancer_guidelines_final_3-25-05.pdf

[62] https://www.epa.gov/sites/production/files/2013-09/documents/cancer_guidelines_final_3-25-05.pdf

historical control data because it can be very misleading and mischaracterizing positive evidence of cancer as negative or no evidence (as in this case). OECD Guidelines stipulate that the use of historical control data may only be appropriate if there is evidence that the data from concurrent controls are "substantially out of line with recent data from control animals from the test facility colony" – there is no documentation of such a problem with the concurrent control data in this study (OECD 2010, Section 5.4.4).[63]

EPA (1993) dismissed cancer evidence at high doses, citing a potential for general toxicity in the highest dose group. Toxicity would present itself as either an increase in mortality in this group and/or a significant reduction in body weight without a significant reduction in food consumption. It is not clear if this was the case – no data was presented to support this claim. Moreover, the liver tumors in male rats were also observed at lower doses, making it biologically plausible that the ones at the highest dose are also treatment related.

The studies for imidacloprid are likely to underestimate human cancer risks because they were only 2-years long, which is equivalent to a person of about 70 years old; this fails to address the fact that most cancers in humans do not occur until after this age. Since about 80% of all human cancers occur in people over the age of 60, even a conventional 2-year bioassay does not have sufficient latency period to detect tumor that will occur later in life.[64] Cadmium is an example of a chemical that was not shown to be carcinogenic in 2-year studies of Wistar rats[65] but caused various tumors in the lung in a 31-month study of Wistar rats.[66] Toluene is another example, Soffritti et al. (2004) orally treated Sprague-Dawley rats with toluene for 2 years and observed them for an additional 6 months. At the end of this observation period, significant increases occurred in several tumors, including total malignant tumors; tumors of subcutaneous tissue, mammary glands, and oral cavity, lips, and tongue; and lymphomas/leukemias.[67] In contrast, the National Toxicology Program's (NTP) 2-year inhalation bioassays in Fischer rats and B6C3F mice did not detect any chemically related tumors.[68] Thus, the 26 extra weeks of observation without additional exposure in the Sprague-Dawley study of Soffritti et al (2004) allowed time for tumors to develop, progress, and become detectable.[69] NIEHS Scientist and former Chief of the IARC Monograph Programme, Dr. James Huff and co-authors concludes that ceasing exposure at 2 years without monitoring tumor development for additional time cannot estimate the impact of food additives, drugs, and other chemicals on humans who die in their

---

[63] http://www.oecd.org/chemicalsafety/testing/44960015.pdf

[64] Huff J, Jacobson MF, Davis DL. The Limits of Two-Year Bioassay Exposure Regimens for Identifying Chemical Carcinogens. Environmental Health Perspectives. 2008;116(11):1439-1442

[65] Löser E. A 2 year oral carcinogenicity study with cadmium on rats. Cancer Lett. 1980;9:191–198

[66] Takenaka S, Oldiges H, Konig H, Hochrainer D, Oberdorster G. Carcinogenicity of cadmium chloride aerosols in W rats. J Natl Cancer Inst.1983;70:367–373

[67] Soffritti M, Belpoggi F, Padovani M, Lauriola M, Degli Esposti D, Minardi F. Life-time carcinogenicity bioassays of toluene given by stomach tube to Sprague-Dawley rats. Eur J Oncol. 2004;9:91–102.

[68] Huff J. Absence of carcinogenic activity in Fischer rats and B6C3F1 mice following 103-week inhalation exposures to toluene. Int J Occup Environ Health. 2003;9:138–146

[69] Soffritti M, Belpoggi F, Padovani M, Lauriola M, Degli Esposti D, Minardi F. Life-time carcinogenicity bioassays of toluene given by stomach tube to Sprague-Dawley rats. Eur J Oncol. 2004;9:91–102.

70s or later.[70] That is why scientists at the National Institutes of Environmental Health Sciences (NIEHS) reject the notion that an 18-month rodent bioassay (about 30-50 years in human age) is long enough to reliably predict cancer risks.[71] Instead many scientists recommend extending the rodent bioassay to 30 months, and including pre-natal exposures.[72]

EPA should re-do its cancer assessment for imidacloprid, and identify its cancer risks consistent with the available evidence, including statistically significant adenoma/carcinomas and statistically significant rare cholangiocellular carcinomas, which is a very aggressive and often deadly cancer in humans.

Chronic/Oncogenic Oral Studies – Mice

EPA (1993) had two chronic oncogenicity oral feeding studies in mice (83-2; MRID 422563-35, 422563-36). The mice studies are so poorly conducted that they should be deemed as impossible to interpret, and not as evidence for lack of carcinogenicity.

Imidacloprid (95.3% a.i.) was administered to B6C3F1 mice (50/sex/dose) for a period of 24 months. The dietary levels were 0, 100, 330 or 1000 ppm (Watta-Gebert, 1991) and 0 and 2000 ppm (Watta-Gebert, 1991a). Ten more mice/sex/dose were used for interim examinations after 12 months of treatment. The two studies had similar protocols and, therefore, were evaluated together. The reported average daily doses were 0, 20, 66, 208 or 414 mg/kg/day for males and 0, 30, 104, 274 and 424 mg/kg/day for females.

Cal DPR[73] reported that the food intake represented about 22-28% of the body weight of an adult mouse – which is a gigantic range, and at the upper end is an inconceivably high amount

---

[70] Huff J, Jacobson MF, Davis DL. The Limits of Two-Year Bioassay Exposure Regimens for Identifying Chemical Carcinogens. Environmental Health Perspectives. 2008;116(11):1439-1442

[71] Bucher JR. The National Toxicology Program rodent bioassay: designs, interpretations, and scientific contributions. Ann NY Acad Sci.2002;982:198–207.
Haseman J, Melnick R, Tomatis L, Huff J. Carcinogenesis bioassays: study duration and biological relevance. Food Chem Toxicol. 2001;39:739–744.
Kodell RL, Lin KK, Thorn BT, Chen JJ. Bioassays of shortened duration for drugs: statistical implications. Toxicol Sci. 2000;55:415–432.

[72] Haseman J, Melnick R, Tomatis L, Huff J. Carcinogenesis bioassays: study duration and biological relevance. Food Chem Toxicol. 2001;39:739–744.
Huff J. Chemicals studied and evaluated in long-term carcinogenesis bioassays by both the Ramazzini Foundation and the National Toxicology Program: in tribute to Cesare Maltoni and David Rall. Ann NY Acad Sci.2002;982:208–230.
Huff J, Lunn RM, Waalkes MP, Tomatis L, Infante PF. Cadmium-induced cancers in animals and in humans. Int J Occup Environ Health.2007;13:202–212
Maltoni C. The contribution of experimental (animal) studies to the control of industrial carcinogenesis. Appl Occup Environ Hyg. 1995;10:749–760.
Soffritti M, Belpoggi F, Minardi F, Maltoni C. Ramazzini Foundation cancer program: history and major projects, life-span carcinogenicity bioassay design, chemicals studied, and results. Ann NY Acad Sci. 2002;982:26–45.

[73] Cal DPR 2006. Risk Characterization Document for Imidacloprid. Available at: http://www.cdpr.ca.gov/docs/risk/rcd/imidacloprid.pdf

of food. Cal DPR proposes that, "these high levels of food consumption (and imidacloprid intake) may be due to not accounting for food spillage in the calculations for the consumed food." (Cal DPR, page 39). This essentially means that the dose per animal is unknown or at best can only be roughly guesstimated. A marked increase in mortality was reported for the males in the 2000 ppm group that died intercurrently (34 % vs. 12% control, p=0.002), which Cal DPR stated was, "due to the large number of males, which died during manipulations such as blood withdrawals, tattooing or as a result of being caught in the automatic feeder. The later was attributed by the authors to the general debilitation and major reduction in body weights caused by imidacloprid" (Cal DPR, page 39). [74] These deaths caste serious doubt on not only the scientific claims of the study, but on the ethical conduct as well – and for good reasons.

EPA (1993) considered the mouse studies to be evidence of lack of carcinogenicity, with a NOAEL of 1000 ppm (208 mg/kg/day in females; 274 mg/kg/day in males), and a LOAEL of 2000 ppm based on decreased body weight, decreased food consumption, and decreased water intake in both sexes.[75] Cal DPR also considered the mouse studies to be evidence of lack of carcinogenicity, but took a more health-protective approach to deriving a risk estimate: "DPR toxicologists adjusted the ingestion of imidacloprid to 1/7 of the mice mean body weight... which is similar to a default food consumption of 15 % of the body weight of an adult mouse. The revised NOEL would be 47 mg/kg/day, based on the revised LOEL of 143 mg/kg/day" (Cal DPR, page 39). [76]

While Cal DPR risk estimates are over 4-fold more health protective, both EPA HHDRA and Cal DPR should not be using such a poorly conducted and unreliable – and likely also unethical - study as affirmative information for lack of carcinogenicity, especially when the individual food consumption and therefore the dose was plucked from thin air, and the mice were being killed with ether, handling, and their own feeding device! Cancer and other chronic disease stood little chance of being noticed amid such seemingly acute mistreatment and protocol mismanagement.

Overall, the mouse study is uninformative of cancer risk, and therefore cannot be used to dismiss affirmative evidence of elevated liver cancer risk from the rat studies.

Chronic/Oncogenic Oral Studies – Dog

EPA (1993) had one chronic and carcinogenic oral feeding study in dogs (MRID 422730-02; Allen et al 1989). Imidacloprid was administered through the diet for a period of 52 weeks to adult

---

[74] Cal DPR 2006. Risk Characterization Document for Imidacloprid. Available at: http://www.cdpr.ca.gov/docs/risk/rcd/imidacloprid.pdf

[75] EPA 1993. ID No 003125-UEE, 003125-UEG, 3F04169, 3H05655. Imidacloprid Evaluation of Toxicity Data Submitted and Identification of Outstanding Toxicology Data Requirements. Tox review 010537. 09/03/1993. Available on Chem Search and at https://www3.epa.gov/pesticides/chem_search/cleared_reviews/csr_PC-129099_3-Sep-93_041.pdf

[76] Cal DPR 2006. Risk Characterization Document for Imidacloprid. Available at: http://www.cdpr.ca.gov/docs/risk/rcd/imidacloprid.pdf

Beagle dogs (4/sex/dietary level) at 200, 500 or 1250 ppm. The 1250 ppm dose was increased to 2500 ppm from week 17 to the end of the treatment. These levels correspond to daily doses of 6, 15 and 41/72 mg/kg/day. The chronic oral NOEL was established by the authors as 500 ppm (15 mg/kg/day), based on liver changes at the LOEL of 1250/2500 ppm (41 mg/kg/day). EPA derived a NOAEL of 1250 ppm and a LOAEL of 2500 ppm, which is 2.5-fold weaker (more permissive) than the NOAEL derived by the authors.

Overall, the dog study is uninformative of cancer risk and therefore cannot be used to dismiss affirmative evidence of elevated cancer risk from other studies.

CONCLUSION:

NRDC makes the following recommendations, based on the scientific record detailed in our comments:

- Consistent with the available evidence, EPA should apply an FQPA factor of at least 10X to account for the evidence of developmental neurotoxicity, including the more severe and even deadly effects in the offspring compared to the less severe maternal effects.
- EPA should classify imidacloprid as an endocrine disruptor, consistent with the affirmative evidence of biologically relevant adverse effects from the EDSP Tier 1 tests.
- EPA should re-do its cancer assessment of imidacloprid to address the evidence of cancer risks, including significant elevate risks of rare and deadly cancers, from guideline studies.
- EPA should cancel all pet collar uses because of the excessive risks they pose to children; risks would be calculated to be even higher if EPA applied the legally-mandated default 10X FQPA factor.

In summary, EPA is in the business of protecting the public, not agrochemical company profits. Dr. Melnick, retired career NIEHS scientist, warned that serious public health consequences may follow if chemicals are misclassified as less toxic or non-toxic based on untested mechanistic hypotheses, poorly validated tests, or incomplete data sets. In his words, "[d]eclaring a chemical as not hazardous, or reducing a level of health protection, should require validation, not speculation."[77]

The warning against false negatives – calling something safe when it is harmful – is echoed by EPA statistician Herbert Lacayo in a recently surfaced internal EPA memo on glyphosate cancer risks. In his 1985 memo dismissing Monsanto's pleas to ignore cancer evidence in rodents treated with glyphosate, Dr. Lacayo wrote, "Our viewpoint is one of protecting the public health when we see suspicious data. It is not our job to protect registrants from false positives."[78] EPA OPP must not lose its way.

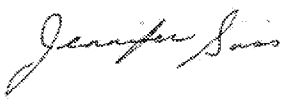
---

[77] Melnick RL, Kamel F, Huff J. Declaring chemicals "not carcinogenic to humans" requires validation, not speculation. Environ Health Perspect. 2003 Apr;111(4):A203-4.

[78] EPA 1985. Memo from Herbert Lacayo, Statistician, to Reto Engler, Chief on the Use of historical data in determining the weight of evidence from kidney tumor incidence in the Glyphosate two-year feeding study; and

Thank you for the opportunity to provide comments.

Respectfully submitted,



Jennifer Sass, Ph.D.
Natural Resources Defense Council, Senior Scientist

Ruth Berlin, Executive Director
Maryland Pesticide Network

Cynthia Palmer,
Director, Pesticides Science and Regulation
American Bird Conservancy

---

some remarks on false positives.
https://archive.epa.gov/pesticides/chemicalsearch/chemical/foia/web/pdf/103601/103601-170.pdf